Exploring the fitness landscape of gastroenteritis-associated and bloodstream infection-associated *Salmonella* Enteritidis

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by

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Abstract

Exploring the fitness landscape of gastroenteritis-associated and bloodstream infection-associated *Salmonella* Enteritidis

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Non-typhoidal *Salmonella* (NTS) typically causes gastroenteritis in humans. However, in recent years NTS have emerged as a major causative agent of bloodstream infections in sub-Saharan Africa, with *Salmonella* Enteritidis ranked as the second most common serovar associated with this invasive disease. Genomic and phylogenetic characterisation of *S*. Enteritidis isolates from human bloodstream has identified the Central/Eastern African clade (CEAC) and Western African clade that are significantly different to the gastroenteritis-associated Global Epidemic clade (GEC). The African clades have distinct genetic features such as genomic degradation, novel prophage repertoires and multi-drug resistance. The molecular basis for the enhanced propensity of African *S*. Enteritidis to cause bloodstream infection remains poorly understood.

This thesis explores the molecular basis for African *S.* Enteritidis virulence, by investigating the genetic determinants of the GEC-representative strain P125109 and the CEAC-representative strain D7795 for growth *in vitro* in LB, NonSPI2 and InSPI2 conditions, and replication in RAW 264.7 murine macrophages. Two functional genomic approaches, RNA sequencing (RNA-seq) and transposon insertion sequencing (TIS), were considered.

Previously, a SDS-phenol-ethanol-based differential lysis approach had proved successful for isolating *Salmonella* RNA from infected eukaryotic cells for RNA-seq analyses. Unfortunately, RNA yields were often low or highly contaminated with eukaryotic RNA. I performed experiments to optimise the separation and recovery of intra-macrophage *Salmonella* and found that needle-and-syringe-mediated lysate homogenisation showed most promise in improving intra-macrophage *Salmonella* recovery, although the associated increase in intra-macrophage *Salmonella* RNA yield proved to be unreproducible. Separately, a comparative analysis of four commonly-used RNA extraction methods showed that the RNA extracted with TRIzol was superior in terms of RNA quality and concentration.

In light of the unresolved technical challenges with intracellular *Salmonella* RNA extraction, TIS was used to investigate the genetic requirements of P125109 and D7795 for *in vitro* growth and macrophage infection. I identified 207 *in vitro*-required genes common to both strains that were also required by other *Salmonella* serovars and *Escherichia coli*, and 63 genes that showed a strain-specific requirement. Distinct patterns for gene requirements for optimal growth in LB, NonSPI2 and InSPI2 were observed between P125109 and D7795, suggesting that the two strains have differing metabolic capabilities.

Screening the transposon libraries during macrophage infection identified about 200 genes belonging to each strain that contribute to bacterial survival and replication in mammalian cells. The majority of the genes have characterised roles in *Salmonella* virulence. I identified candidate strain-specific virulence genes, including genes with no known orthologues in the other *Salmonella* strains referenced in this study, representing potential novel *Salmonella* virulence factors.

I anticipate that my TIS findings will serve as a useful community resource for investigation of *S.* Enteritidis gene functions. Future integrative analyses of my TIS-based fitness data with other “-omics” datasets should yield new insights into African *S.* Enteritidis infection biology.

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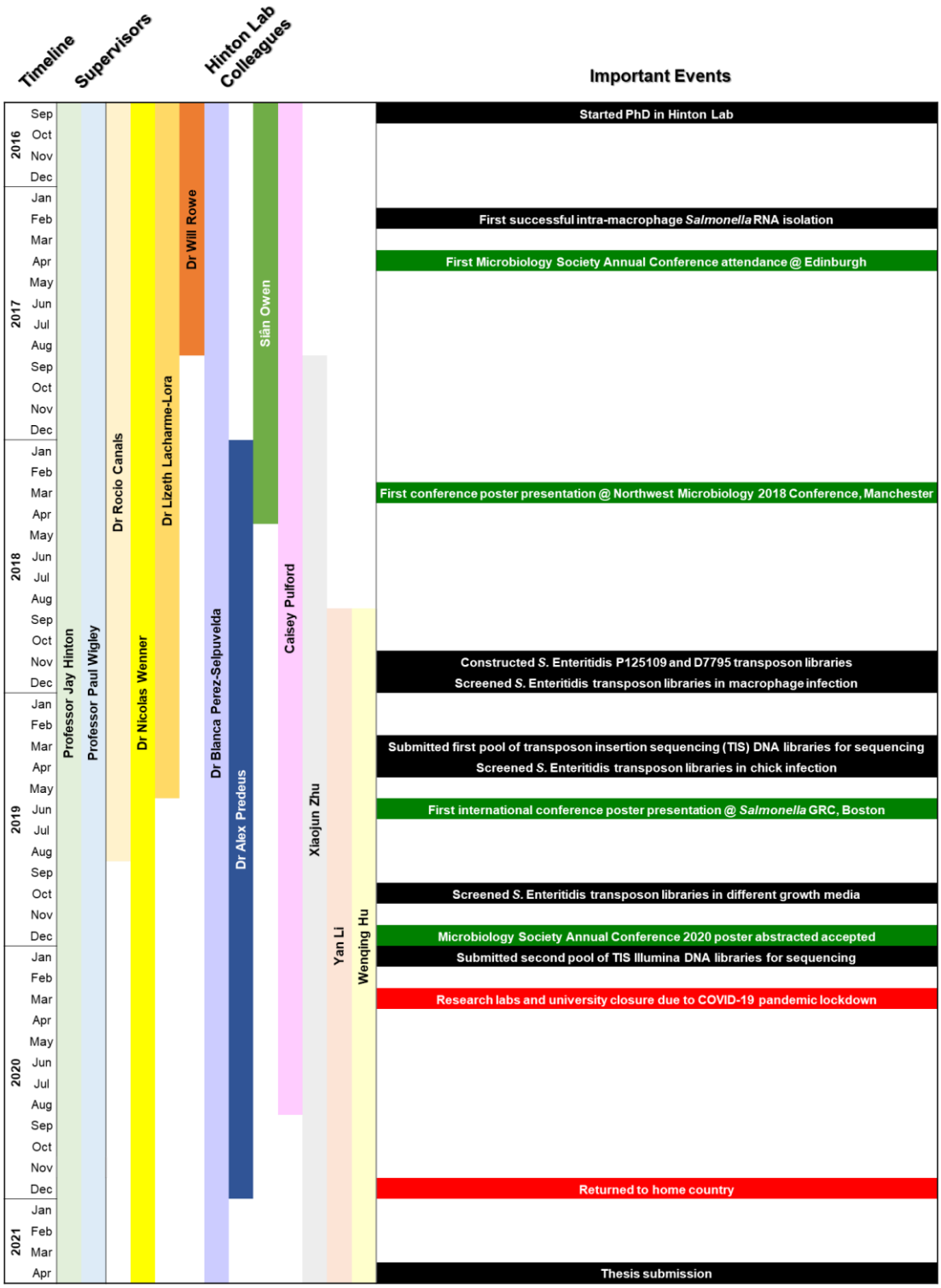
My PhD Journey



Publications and Conference Presentations

Conference presentations resulting from work described in this thesis

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Poster presentation: **Identification of genes that contribute to fitness of African and global clades of *Salmonella* Enteritidis during infection of macrophages**

June 2019 ECM Forum Summer Conference 2019, Dublin, Ireland

Poster presentation: **Identification of genes that contribute to fitness of African and global clades of *Salmonella* Enteritidis during infection of macrophages**

Co-authored publications during the duration of this PhD

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List of Abbreviations

Ab	Antibiotic
Amp	Ampicillin
CFU	Colony forming units
Cm	Chloramphenicol
COG	Cluster of Orthologous Genes
DMB	Density marker bead
DMEM	Dulbecco's Modified Eagle's medium
DPBS	Dulbecco’s phosphate buffered saline
ESP	Early stationary phase
EtOH	Ethanol
FBS	Foetal bovine serum
GFP	Green Fluorescent Protein
Gm	Gentamicin
HITS	High-throughput insertion tracking by deep sequencing
HPLC	High performance liquid chromatography
HPSF	High-Purity, Salt-Free
INSeq	Insertion sequencing
Km	Kanamycin
LB	LB-Lennox (5 g/L NaCl)
MEM NEAA	MEM Non-essential amino acids
MilliQ H_2O	Ultrapure type I water with a resistivity of 18.2 MΩ.cm at 25°C
MLNs	Mesenteric lymph nodes
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NMS	Normal mouse serum
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCN	Phosphate-carbon-nitrogen
PCR	Polymerase chain reaction
PES	Polyethersulfone

PPs	Peyer’s patches
RDAR	Red, dry and rough
RNA-seq	RNA sequencing
ROD	Region of Difference
RT-qPCR	Quantitative reverse transcription PCR
SCV	*Salmonella*-containing vacuole
SNP	Single nucleotide polymorphism
SPI	*Salmonella* Pathogenicity Island
sRNA	Small RNA
ST	Sequence type
STM	Signature-tagged mutagenesis
T1SS	Type I secretion system
T6SS	Type VI secretion system
TB	Terrific broth
Tet	Tetracycline
TIS	Transposon insertion sequencing
TMDH	Transposon-mediated differential hybridisation
Tn	Transposon
Tn-Seq	Transposon sequencing
TraDIS	Transposon-directed insertion site sequencing
TraSH	Transposon site hybridisation
tRNA	Transfer RNA
TTSS	Type III secretion system
TTSS-1	Type III secretion system encoded by SPI-1
TTSS-2	Type III secretion system encoded by SPI-2

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Contents of accompanying Excel spreadsheets

Chapter 1 :

Introduction

1.1 Overview of the *Salmonella* genus

Salmonella is a genus of Gram-negative, facultatively anaerobic rod-shaped bacteria that belongs to the Enterobacteriaceae family (Eng *et al.*, 2015). The genus comprises two species, *S. bongori* and *S. enterica*, the latter of which is divided into six subspecies (denoted by Roman numerals): I, subsp. *enterica*; II, subsp. *salamae*; IIa, subsp. *arizona*; IIb, subsp. *diarizonae*; IV, subsp. *houtena*; and VI, subsp. *indica* (Ryan *et al.*, 2017). Currently, there are 2,579 serovars of *Salmonella*, based on the White-Kauffmann-Le Minor scheme that distinguishes salmonellae according to their surface somatic O and flagellar H antigens (Grimont and Weill, 2007). 1,531 serovars (59%) belong to *S. enterica* subspecies I, which are found predominantly in mammals and causes infections in humans and warm-blooded animals (Brenner *et al.*, 2000). *S. bongori* and the other *S.* enterica subspecies are found principally in cold-blooded animals and the environment but rarely isolated from humans (Brenner *et al.*, 2000).

Salmonella serovars can be classified based on their host range as being either host-restricted, host-adapted or host-generalist (Uzzau *et al.*, 2000). *Salmonella* serovars are also categorised according to their clinical manifestations in human disease as typhoidal *Salmonella* and non-typhoidal *Salmonella* (NTS). Typhoidal *Salmonella* refers to the serovars *Salmonella enterica* subsp. *enterica* Typhi (*S.* Typhi) and *Salmonella enterica* subsp. Paratyphi (*S.* Paratyphi), which exclusively infect humans and cause typhoid fever and paratyphoid fever respectively, collectively known as enteric fever. Enteric fever is a systemic invasive illness characterised by fever fluctuations, headache, abdominal pain, and transient diarrhoea or constipation, and can cause fatal respiratory, hepatic, spleen, and/or neurological damage (Hiyoshi *et al.*, 2018). The disease is endemic in low-income settings where clean water and adequate sanitation is lacking (Gal-Mor *et al.*, 2014). Recent estimates of global disease burden show there are approximately 12 million cases and 128,000 deaths per year (Mogasale *et al.*, 2014).

NTS serovars, on the other hand, usually cause self-limiting gastroenteritis in humans, an inflammatory condition of the gastrointestinal tract that is accompanied by symptoms such as non-bloody diarrhoea, vomiting, nausea, headache, abdominal cramps and myalgias (muscle aches), and which resolves naturally in immunocompetent individuals (Eng *et al.*, 2015). Notable NTS serovars that cause disease in humans are *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis), which are host-generalists and can infect multiple hosts including mice, poultry, and pigs. Incidence and mortality estimates in 2010 showed that *Salmonella*-associated gastroenteritis accounted for 93.8 million cases of illness and 155,000 deaths per year (Majowicz *et al.*, 2010).

In about 5% of NTS infections, NTS bacteria can cause an invasive, extra-intestinal disease leading to bacteraemia and focal systemic infections in humans, hereafter referred to as iNTS disease (Gal-Mor *et al.*, 2014). iNTS infection in humans can be caused by host-adapted *Salmonella* serovars which are usually found in animals e.g. *Salmonella enterica* subsp. Dublin (*S.* Dublin; host-adapted to cattle) and *Salmonella enterica* subsp. Choleraesuis (*S.* Choleraesuis; host-adapted to pigs), or genetic variants of *S.* Typhimurium and *S.* Enteritidis identified in sub-Saharan Africa (further discussed in Section 1.5). iNTS disease usually manifests as a febrile systemic illness resembling enteric fever that lacks gastrointestinal symptoms, and in recent years has emerged as the most common bloodstream infection in sub-Saharan Africa (Marchello *et al.*, 2020; Reddy *et al.*, 2010).

1.2 Overview of the *Salmonella* infection process

Salmonella infection process begins with the ingestion of contaminated food or water. Upon reaching the stomach, *Salmonella* activates the acid tolerance response (ATR) (reviewed in Álvarez-Ordóñez *et al.*, 2011) to survive the acidic conditions. After passing through the stomach and reaching the intestinal tract, *Salmonella* adheres to and invades the intestinal epithelium. Invasion usually occurs via the M cells of the Peyer's patches (PPs) in the intestinal epithelium (although invasion of non-phagocytic enterocytes can also happen), and involves cytoskeletal rearrangements in the host cells leading to formation of membrane ruffles that engulf the bacteria into large vesicles called *Salmonella*-containing vacuoles (SCVs) (Fàbrega and Vila, 2013; Shah *et al.*, 2017). Invasion of the intestinal epithelium also triggers an inflammatory response, which results in the recruitment and transmigration of phagocytes from the submucosal space into the intestinal lumen and production of pro-inflammatory cytokines (Fàbrega and Vila, 2013).

Intracellular *Salmonella* resides within the SCV, where bacterial replication takes place. In systemic *Salmonella* infections, the SCVs are released on the basolateral side of the intestinal epithelium, where the bacteria can be engulfed by neutrophils, macrophages or dendritic cells. Following phagocytosis-mediated uptake by macrophages, *Salmonella* can survive and replicate within SCVs, and spread systemically (Petersen and Miller, 2019). Since *Salmonella* preferentially invades M cells of PPs, which are organised lymphoid follicles, the infected phagocytes transport the bacteria to the mesenteric lymph nodes (MLNs) where *Salmonella* can be further disseminated via the bloodstream to additional tissues, particularly the liver and spleen (Fàbrega and Vila, 2013).

1.3 *Salmonella* Enteritidis

S. Enteritidis is the one of the most common serovars isolated from human NTS infections worldwide, and is the focal point of research in this PhD study. Since the mid-1980s, *S*. Enteritidis has caused a global pandemic of foodborne illness associated with contaminated eggs and poultry (Rodrigue *et al.*, 1990). Analysis of data on *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank showed that *S.* Enteritidis accounted for 65% and 43% of NTS infections globally in 2002 and 2007 respectively (Galanis *et al.*, 2006; Hendriksen *et al.*, 2011). In both studies, *S.* Enteritidis ranked as the most common serovar in all regions apart from the Oceania and North American regions, where *S.* Typhimurium was the most common serovar reported.

One reason for the high incidence of human disease mediated by *S.* Enteritidis is that this serovar can cause asymptomatic infections in chickens and can be transmitted to eggs via the ovarian tract, and the bacteria can survive and persist within eggs (reviewed in Gantois *et al.*, 2009). Eggs and egg products are among the highest risk foods in *Salmonella* outbreaks, as exemplified by the recent *S.* Enteritidis outbreak that began in Poland in 2016 and continued in 2017 (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018; Pijnacker *et al.*, 2019). A comprehensive review on the mechanisms used by *S.* Enteritidis to colonise chickens is beyond the scope of this thesis, but has been covered elsewhere (De Buck *et al.*, 2004; Foley *et al.*, 2013; Gantois *et al.*, 2009).

1.4 *Salmonella* virulence strategies and mechanisms

Salmonella possesses many virulence strategies for interacting with the host during the infection process. The following sections will briefly discuss the most pertinent virulence strategies used by *Salmonella*, drawing on studies on both *S.* Enteritidis and *S.* Typhimurium.

1.4.1 *Salmonella* Pathogenicity Islands

Salmonella Pathogenicity Islands (SPIs) are large, distinct genetic cassettes within the *Salmonella* chromosome that harbour virulence determinants (Hensel, 2009; Marcus *et al.*, 2000). Like other pathogenicity islands, SPIs generally have a lower GC content compared with the overall bacterial genome, and are often associated with genes encoding transfer RNAs (tRNAs), suggesting that they are horizontally acquired (Hensel, 2009). To date, 23 SPIs have been identified, of which SPI-1 to SPI-5 are present in all *Salmonella enterica* serovars while the others are variably distributed (Wang *et al.*, 2020).

1.4.1.1 SPI-1

SPI-1 is approximately 40 kb in size and is primarily required for the invasion of the intestinal epithelium (Lou *et al.*, 2019). The genes within SPI-1 encode various components of a type III secretion system (TTSS), its regulators and secreted effectors. TTSS are molecular syringes that deliver effector proteins into host cells during infection to modulate host cell activity and aid the infection process (dos Santos *et al.*, 2020). Upon host cell contact, the SPI-1 TTSS (TTSS-1) is expressed and delivers effector proteins into host cells to induce membrane ruffling, where the cell membrane extends outwards and engulfs *Salmonella* (Foley *et al.*, 2013; Marcus *et al.*, 2000). SPI-1 also induces neutrophil recruitment, leading to a reduction and alteration in intestinal microbiota (Lou *et al.*, 2019). In both *S.* Enteritidis and *S.* Typhimurium, SPI-1 genes have been shown to be required for the invasion of

intestinal epithelial cells *in vitro* and systemic spread to the liver and spleen in chicken and mice infection *in vivo*, but do not seem to be required for intestinal colonisation (Desin *et al.*, 2009; Lawley *et al.*, 2006; Rychlik *et al.*, 2009; Silva *et al.*, 2012).

1.4.1.2 SPI-2

SPI-2 consists of two regions, a smaller 14.5 kb region containing five *ttr* genes involved in tetrathionate reduction (Hensel *et al.*, 1999a) and seven open reading frames (ORFs) of unknown function, and a larger 25.3 kb portion containing genes encoding a second TTSS (TTSS-2) (Hensel *et al.*, 1999b).

The *ttr* genes contribute to the enteric phase of *Salmonella* infection by conferring *Salmonella* the ability to use tetrathionate, a respiratory electron acceptor generated as a result of the inflammatory response, thereby allowing *Salmonella* to outgrow the microbiota in the lumen of the inflamed gut (Winter *et al.*, 2010a). A recent report indicates that *ttrA* is not required by *S*. Enteritidis and *S.* Typhimurium for caecal colonisation or systemic infection in chickens (Saraiva *et al.*, 2021).

The TTSS-2 is crucial to the ability of *Salmonella* to survive inside infected cells and contributes to systemic dissemination (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996). Expression of the SPI-2 genes is highly upregulated following internalisation of *Salmonella* into epithelial cells or macrophages, and coincides with the formation of the SCV (Eriksson *et al.*, 2003; Hautefort *et al.*, 2008).

1.4.1.3 SPI-3

SPI-3 is a 17 kb island containing at least ten genes characterised into six transcriptional units (Blanc-Potard *et al.*, 1999), of which only four ORFs have been functionally characterised. *mgtCB* are required for intra-macrophage survival and virulence in mice (Blanc-Potard *et al.*, 1999; Blanc-Potard and Groisman, 1997). MisL

is an autotransporter protein that contributes to intestinal colonisation in mice (Dorsey *et al.*, 2005), whose expression is activated by MarT (Tükel *et al.*, 2007). In *S.* Enteritidis, SPI-3 genes appear to be important for systemic infection in mice but not in chickens (Rychlik *et al.*, 2009; Silva *et al.*, 2012).

1.4.1.4 SPI-4

SPI-4 is a 27 kb region that carries six genes designated *siiABCDEF* (McClelland *et al.*, 2001; Wong *et al.*, 1998). *siiCDF* encodes a type I secretion system (T1SS), which secretes SiiE, a giant nonfimbrial adhesin that mediates close interaction with the intestinal epithelium and facilities invasion (Gerlach *et al.*, 2007; Li *et al.*, 2019; Morgan *et al.*, 2007). The SPI-4 genes are important for *S.* Enteritidis and *S.* Typhimurium intestinal colonisation of mice, but do not appear to be important for chicken colonisation (Kiss *et al.*, 2007; Morgan *et al.*, 2004; Rychlik *et al.*, 2009). SiiE also has a reported role in long term persistence in mice, by interfering with the humoral immune response to prevent *Salmonella* clearance (Lawley *et al.*, 2006; Männe *et al.*, 2019).

1.4.1.5 SPI-5

SPI-5 was first identified in *S.* Dublin and is required for enteric but not systemic infection (Wood *et al.*, 1998). The SPI-5 island is approximately 7 kb in size and encodes at least five genes: *pipA*, *pipB*, *pipC*, *pipD*, and *sopB*. The encoded proteins are related to the intestinal mucosal fluid secretion and inflammatory responses, and are regulated by TTSS-1 and TTSS-2 (Knodler *et al.*, 2002; Wang *et al.*, 2020). In *S.* Enteritidis, the SPI-5 genes have been reported to contribute to systemic infection of mice but not chickens (Rychlik *et al.*, 2009; Silva *et al.*, 2012).

1.4.1.6 Other *S.* Enteritidis SPIs with role in virulence

In addition to the five major SPIs, several other SPIs have been shown to specifically contribute to *S.* Enteritidis virulence. Espinoza *et al.* (2017) reported that SPI-13 was required for internalisation of *S.* Enteritidis in murine but not human macrophages. Several genes within SPI-19, also known as Region of Difference (ROD) 9 (Thomson *et al.*, 2008), have been shown to have a role in infection of cultured cells and animals: Silva *et al.* (2012) demonstrated that *SEN1001* is required for liver and spleen colonisation in mice, and survival and replication in RAW 264.7 murine macrophages, and Das *et al.* (2018; 2020) showed that *SEN1005* and *SEN1008* contribute to murine infection. The SPI-19 region in *S.* Enteritidis is missing a 24 kb region that is present in *S. enterica* subsp. *enterica* Gallinarum (*S.* Gallinarum), an avian-restricted serovar that causes fowl typhoid, and encodes remnant components of a type VI secretion system (T6SS) (Blondel *et al.*, 2009). Interestingly, Blondel *et al*. (2010) observed that transferring the intact T6SS encoded by the *S.* Gallinarum SPI-19 region to *S.* Enteritidis reduces the ability of *S.* Enteritidis to colonise chickens.

1.4.2 Effector proteins

Effector proteins are small molecules secreted by *Salmonella* into host cells during infection to modulate host activity, and are translocated by either the TTSS-1 or TTSS-2. To date, over 40 SPI-1 and SPI-2 effectors have been identified in *S.* Typhimurium (Ramos-Morales, 2012). These effectors play diverse roles during *Salmonella* infection, including manipulation of the host cytoskeleton and subversion of immune signalling, intracellular trafficking, and cell survival pathways (Johnson *et al.*, 2018; Ramos-Morales, 2012). Comparative analysis of *S.* Enteritidis and *S.* Typhimurium effectors showed that the majority of the *S.* Typhimurium effectors are also present in *S.* Enteritidis (Y. Li, personal communication) (**Table 1.1**). A new comparison between *S.* Enteritidis gastroenteritis-associated isolates and iNTS disease-associated isolates from sub-Saharan Africa (Feasey *et al.*, 2016) revealed

distinct differences in five effectors (SifB, SopB, SptP, SpvD and SseK3) (**Figure 1.1**), all of which have reported roles in NF-κB signalling modulation (Johnson *et al.*, 2018). The functions of the other effector proteins listed in **Table 1.1** are reviewed in Jennings *et al.* (2017) and Johnson *et al.* (2018).

Table 1.1 The type III secretion system (TTSS) effector repertoire of *S.* Typhimurium and *S.* Enteritidis[a]

TTSS	Effector	Genomic location	*S.* Typhimurium LT2	*S.* Enteritidis P125109[b]	*S.* Enteritidis P125109 (new)[c]	*S.* Enteritidis D7795 (new)[c]
SPI-1	SopB	SPI-5	STM1091	SEN0955	P125109_01071	D7795_01154
	SopA		STM2066	SEN2065	P125109_02346	D7795_02393
	SipA	SPI-1	STM2882	SEN2723	P125109_03096	D7795_03194
	SipB	SPI-1	STM2885	SEN2726	P125109_03100	D7795_03198
	SipC	SPI-1	STM2884	SEN2725	P125109_03099	D7795_03197
	SipD	SPI-1	STM2883	SEN2724	P125109_03098	D7795_03196
	SptP	SPI-1	STM2878	SEN2720	P125109_03092	D7795_03190
	SopD		STM2945	SEN2784	P125109_03165	D7795_03263
	SopE	SopEϕ	Absent	SEN1155	P125109_01298	D7795_01380
	SopE2	Bacterio-phage remnant	STM1855	SEN1182	P125109_01333	D7795_01414
	SopF		STM1239	SEN1810	P125109_02049	D7795_02187
SPI-2	SseF	SPI-2	STM1404	SEN1641	P125109_01849	D7795_01935
	SseG	SPI-2	STM1405	SEN1640	P125109_01848	D7795_01934
	SteD		STM2139	SEN2134	P125109_02425	D7795_02473
	GogB	Gifsy-1	STM2584	Absent	Absent	Absent
	SseK1		STM4157	SEN3941	P125109_04485	D7795_04581
	SseK2		STM2137	Absent	Absent	Absent
	SseK3	ST64B	Absent	SEN1920	P125109_02176	Absent
	SteC		STM1698	SEN1335	P125109_01508	D7795_01589
	PipB	SPI-5	STM1088	SEN0952	P125109_01068	D7795_01151
	PipB2		STM2780	SEN2624	P125109_02976	D7795_03070
	SifA		STM1224	SEN1825	P125109_02065	D7795_02203
	SifB		STM1602	SEN1454	P125109_01640	D7795_01725
	SopD2		STM0972	SEN0876	P125109_00989	D7795_01014
	SpiC	SPI-2	STM1393	SEN1652	P125109_01860	D7795_01946
	SseB	SPI-2	STM1398	SEN1647	P125109_01855	D7795_01941
	SseC	SPI-2	STM1400	SEN1645	P125109_01853	D7795_01939
	SseD	SPI-2	STM1401	SEN1644	P125109_01852	D7795_01938
	SseE	SPI-2	STM1402	SEN1643	P125109_01851	D7795_01937
	SseI	Gifsy-2	STM1051	SEN0916	P125109_01030	D7795_01113
	SseJ		STM1631	SEN1422	P125109_01605	D7795_01689
	SseL		STM2287	SEN2269	P125109_02578	D7795_02626
	SspH2	Bacterio-phage remnant	STM2241	SEN2224	P125109_02527	D7795_02575
	SpvB	pSLT	pSLT039	SEN_p0032	P125109_05005	D7795_05103
	SpvC	pSLT	pSLT038	SEN_p0031	P125109_05006	D7795_05102
	SrfJ		STM4426	Absent	Absent	Absent

Table 1.1 *(continued)* The TTSS effector repertoire of *S*. Typhimurium and *S.* Enteritidis[a]

TTSS	Effector	Genomic location	*S.* Typhimurium LT2	*S.* Enteritidis P125109[b]	*S.* Enteritidis P125109 (new)[c]	*S.* Enteritidis D7795 (new)[c]
SPI-2	CigR	SPI-3	STM3762	SEN3584	P125109_04062	D7795_04161
	GtgA	Bacterio-phage (Gifsy-2)	STM1026	SEN1143	P125109_01286	D7795_01369
SPI-1 or SPI-2	AvrA	SPI-1	STM2865	SEN2707	P125109_03073	D7795_03171
	SlrP		STM0800	SEN0745	P125109_00839	D7795_00864
	SteA		STM1583	SEN1472	P125109_01661	D7795_01747
	SteB		STM1629	SEN1423A	P125109_01607	D7795_01691
	SteD		STM2139	SEN2134	P125109_02425	D7795_02473
	SteE	Bacterio-phage (Gifsy-1)	STM2585	Absent	Absent	Absent
	SpvD	pSLT	pSLT037	SEN_p0030	P125109_05007	D7795_05101
	GtgE	Bacterio-phage (Gifsy-2)	STM1055	SEN0920	P125109_01034	D7795_01117
Unknown	PipA	SPI-5	STM1087	SEN0951	P125109_01067	D7795_01150
	GogA		STM2614	Absent	Absent	Absent
	SssA		STM0359	SEN0342	P125109_00387	D7795_00390
	SssB		STM1478	SEN1571	P125109_01770	D7795_01856
Candidate[d]	YiiQ		STM4082	SEN3872	P125109_04401	D7795_04498
	-		STM1809	SEN1228	P125109_01384	D7795_01465
	PdgL		STM1599	SEN1456	P125109_01644	D7795_01729

[a] Adapted and extended from Johnson *et al.* (2018): this analysis was completed by Dr Alex Predeus and Yan Li. Grey box indicates the absence of the effector protein.

[b] Refers to genome sequence published in Thomson *et al.* (2008) (NCBI accession no. NC_011294.1).

[c] Refers to recently published annotated genome sequences (Perez-Sepulveda *et al.*, 2021) which are used as reference genomes in this thesis.

[d] Predicted in Niemann *et al.* (2011).

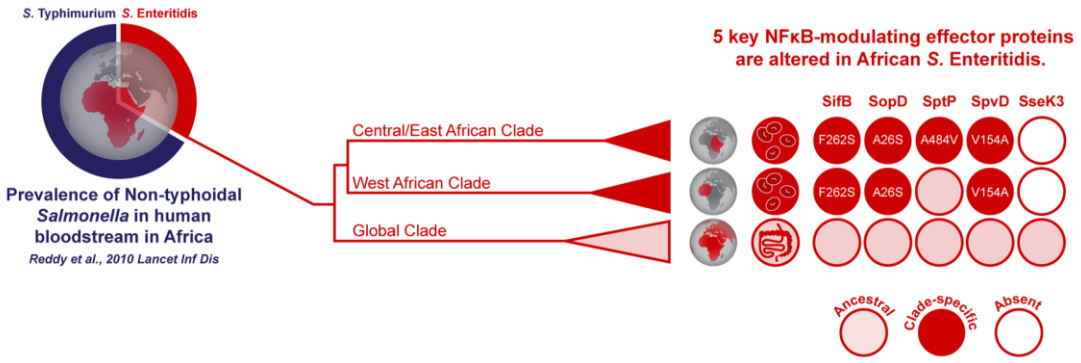

Figure 1.1 Comparative analysis of effector proteins in gastroenteritis-associated and invasive *S.* Enteritidis

The Global Epidemic clade, Central/Eastern clade and West African clade of *S.* Enteritidis are described in Feasey *et al.* (2016), and in Section 1.5. SseK3 is absent from both African clades of *S.* Enteritidis. Clade-specific amino acid substitutions are indicated. This comparative analysis of *S.* Enteritidis effector proteins was completed by Yan Li, and figure was made by Eliza Wolfson.

1.4.3 Fimbriae and flagella

Fimbriae are proteinaceous surface appendages that mediate interactions between bacteria and host cells (Wagner and Hensel, 2011). *S.* Enteritidis and *S.* Typhimurium share in common ten chromosomally-encoded fimbrial clusters (*bcf*, *sti*, *stf*, *stb*, *saf*, *fim*, *csg*, *std*, *lpf* and *sth*) and one plasmid-encoded *pef* operon; the operons *peg*, *ste* and *sef* are unique to *S.* Enteritidis while *stc* and *stj* operons are only found in *S.* Typhimurium (Clayton *et al.*, 2008; Rehman *et al.*, 2019; Thomson *et al.*, 2008). Several *in vitro* and *in vivo* studies have shown that fimbriae are important for adherence of both *S.* Enteritidis and *S.* Typhimurium to epithelial cells and intestinal colonisation (Bäumler *et al.*, 1996b; Bäumler *et al.*, 1996a; Chaudhuri *et al.*, 2013; Clayton *et al.*, 2008; De Buck *et al.*, 2004; Dibb-Fuller *et al.*, 1999; Hansmeier *et al.*, 2017; Thiagarajan *et al.*, 1996). There is also evidence for a role for the *S.* Enteritidis *sef* and *peg* fimbriae in systemic infection in mice (Edwards *et al.*, 2000; Silva *et al.*, 2012); similarly several *S.* Typhimurium fimbriae operons have been implicated in virulence and long term persistence in murine infection (Lawley *et al.*, 2006; Weening *et al.*, 2005).

Flagella are another group of surface appendages that are typically required for motility and chemotaxis (Fàbrega and Vila, 2013). Various studies have reported that flagella play a role in host cell adhesion and invasion, although motility and chemotactic functions may not always be involved or required (Achouri *et al.*, 2015; Allen-Vercoe and Woodward, 1999; Barbosa *et al.*, 2017; Robertson *et al.*, 2003; Van Asten *et al.*, 2000). Flagella are also important activators of host immune responses (Hajam *et al.*, 2017). Extracellular flagella activate toll-like receptor 5 (TLR5) and trigger a signalling cascade that stimulates a proinflammatory response involving the induction of cytokines such as TNF (Hajam *et al.*, 2017; Kawai and Akira, 2011). In contrast, soluble flagellin in the cytosol induces pyroptotic cell death in a caspase-1-dependent manner through activation of the NOD-like receptor NLRC4 (Hajam *et al.*, 2017; Kawai and Akira, 2011; Miao and Rajan, 2011).

The relevance of this immunomodulatory potential is evident from reports that flagella expression is downregulated in *S.* Enteritidis following colonisation of the chicken oviduct and within primary chicken oviduct epithelial cell cultures. It has been proposed that this reduced expression of flagella contributes to the persistent colonisation of the chicken oviduct by *S.* Enteritidis, without concomitant stimulation of an inflammatory response (Kilroy *et al.*, 2017). Similarly, *S.* Typhimurium genetic variants associated with iNTS infection in sub-Saharan Africa express reduced levels of flagellin expression, which may contribute to a ‘stealth’ phenotype during infection and permit higher levels of intracellular survival and replication (Ramachandran *et al.*, 2015). Furthermore, *S.* Typhimurium bacteria that constitutively express flagella were demonstrated to be attenuated during systemic infection (Miao *et al.*, 2010).

1.4.4 Virulence plasmids

S. Enteritidis is among the few *S. enterica* subsp. I serovars that harbours a virulence plasmid (Rotger and Casadesús, 1999; Rychlik *et al.*, 2006). While there are serovar- and strain-specific differences in plasmid composition, the *spv* (*Salmonella* plasmid virulence) operon is typically carried by all *Salmonella* virulence plasmids and is the most well-characterised plasmid-encoded virulence determinant (Rychlik *et al.*, 2006). The *spv* operon contains five genes *spvRACBD*, and is required for *Salmonella* intracellular survival and growth and macrophage killing (Guiney and Fierer, 2011). Expression of the *spv* operon of *S.* Typhimurium is induced by the intracellular environment such as growth restriction, nutrient starvation or lowered pH (Eriksson *et al.*, 2003; Rychlik *et al.*, 2006). Transcription of the *spvACBD* genes is positively controlled by the upstream transcriptional regulatory protein SpvR and requires the alternative sigma factor RpoS for efficient expression (Guiney and Fierer, 2011). SpvA was recently identified as a negative regulator of the *spv* operon (Passaris *et al.*, 2018). SpvB is an ADP-ribosylating enzyme that destabilises host cell actin filaments and mediates cytotoxicity in macrophages (Lesnick *et al.*, 2001), while both SpvC and SpvD have immunomodulatory functions. SpvC is a phosphothreonine lyase that acts on host mitogen-activated protein kinases (MAPKs), and more recently has been implicated in the reverse transmigration of infected dendritic cells into the bloodstream, thereby promoting early extra-intestinal dissemination (Arbibe *et al.*, 2007; Gopinath *et al.*, 2019; Mazurkiewicz *et al.*, 2008). SpvD acts as a serine protease that inhibits NF-κB signalling (Grabe *et al.*, 2016; Rolhion *et al.*, 2016), both of which result in a suppression of the inflammatory response and may contribute to *Salmonella* systemic infection (Passaris *et al.*, 2018). However, the function of the *spv* operon in *S.* Enteritidis has not been studied.

1.4.5 *S.* Enteritidis-specific genes

Comparative genomic analyses of *S.* Enteritidis and other serovars have revealed RODs present in *S.* Enteritidis but not *S.* Typhimurium (Thomson *et al.*, 2008). Recent studies have identified novel virulence genes located in these RODs e.g. the ROD9 genes as discussed in Section 1.4.1.6. In ROD34, the gene *SEN3897*, encoding an alanine racemase, was shown to be important for *in vitro* and *in vivo* invasion and survival in cultured epithelial cells and macrophages and mice (Ray *et al.*, 2018). Another ROD, ROD21, was identified as required for systemic infection in mice (Silva *et al.*, 2012), and has since been shown that excision of this island *in vivo* is required by *S*. Enteritidis to cross the intestinal barrier in mice (Pardo-Roa *et al.*, 2019).

1.5 iNTS disease in sub-Saharan Africa

In recent years, NTS have emerged as the most common causative agent of community-onset bloodstream infections in sub-Saharan Africa (Marchello *et al.*, 2020; Reddy *et al.*, 2010). As mentioned previously, iNTS disease usually manifests as a febrile systemic illness resembling enteric fever that lacks gastrointestinal symptoms. iNTS disease disproportionately affects young children (under the age of five) or individuals with co-morbidities such as malnutrition, malaria or HIV infection (Ao *et al.*, 2015; Feasey *et al.*, 2012; Stanaway *et al.*, 2019; Uche *et al.*, 2017). In 2017, iNTS infection was responsible for 59,100 deaths globally, of which 49,600 deaths occurred in sub-Saharan Africa (Stanaway *et al.*, 2019). The high case fatality-rate of of iNTS (14.5%; Stanaway *et al.*, 2019) makes the disease a major health problem.

Most cases of human iNTS infections across Africa are caused by *S.* Typhimurium or *S*. Enteritidis that are multi-drug resistant, and have distinct genetic signatures from gastroenteritis-associated *Salmonella* (Feasey *et al.*, 2016; Kingsley *et al.*, 2009; Marchello *et al.*, 2020; Reddy *et al.*, 2010). Molecular typing of African invasive

S. Typhimurium isolates (using a technique known as multi-locus sequence typing [MLST]; reviewed in Maiden, 2006) showed that these isolates belong to the novel MLST sequence type 313 (ST313), which is distinct from ST19 that includes most gastroenteritis-associated *S.* Typhimurium (Kingsley *et al.*, 2009). African *S.* Typhimurium ST313 can be further divided into lineages I, II (and sub-lineage II.1) and III (Kingsley *et al.*, 2009; Pulford *et al.*, 2020; Van Puyvelde *et al.*, 2019). Lineage II strain D23580 is generally used as the reference strain for ST313 (Canals *et al.*, 2019b; Kingsley *et al.*, 2009).

iNTS-associated *S.* Enteritidis has been studied less intensively than S. Typhimurium ST313. Feasey *et al.* (2016) identified two separate clades of African invasive *S.* Enteritidis, designated Central/Eastern African and West African clades, which are distinct from the Global Epidemic clade that includes *S.* Enteritidis isolates generally associated with gastroenteritis (**Figure 1.2A**). Global Epidemic strain P125109 and Central/Eastern African strain D7795 are generally used as the reference strains for the respective clades; for brevity, the Central/Eastern African strain D7795 is simply referred to as African strain D7795 for the rest of this thesis.

1.5.1 Comparative genomics of *S.* Enteritidis P125109 and D7795

P125109 was isolated from an outbreak of human food poisoning in the United Kingdom in 1988 (Barrow, 1991; Barrow and Lovell, 1991; Thomson *et al.*, 2008). D7795 was isolated from blood cultures of a Malawian child in 2000 (Feasey *et al.*, 2016). Whole-genome-based sequence comparisons between the two strains revealed genomic degradation and differences in accessory genomes (Perez-Sepulveda *et al.*, 2021) (**Figure 1.2B**). The core genomes of P125109 and D7795 differ by 1,022 single nucleotide polymorphisms (SNPs) (Feasey *et al.*, 2016; Perez-Sepulveda *et al.*, 2021).

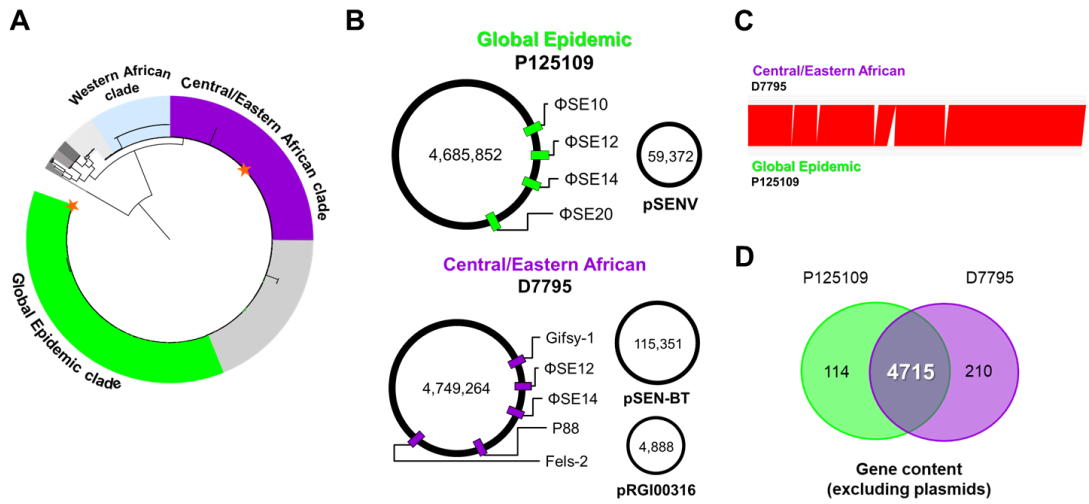

Figure 1.2 Genome comparisons of *S.* Enteritidis P125109 and D7795

A. Phylogenetic tree from Feasey *et al.* (2016) showing the Global Epidemic clade (green), Central/Eastern African clade (purple), and Western African clade (blue); the two orange stars represent the representative strains P125109 and D7795 from the respective clades. **B.** Genome organisation of P125109 and D7795, with the prophage repertoire in each *S.* Enteritidis strain shown. **C.** Alignment of P125109 and D7795 genomes, showing extensive synteny and collinearity. Prophage regions mark the differences between the two genomes. **D.** Comparison of all chromosomally-encoded genes between P125109 and D7795. More than 50% of the genes specific to each strain are prophage genes (60 out of 114 genes in P125109; 164 out of 210 genes in D7795) (**Appendix 1**). Reconstruction of the phylogenetic tree and genome comparisons were completed by Dr Blanca Perez-Sepulveda; complete annotated genomes of P125109 and D7795 are available at BioSample accession numbers SAMN16552336 (D7795) and SAMN16552335 (P125109) (Perez-Sepulveda *et al.*, 2021).

1.5.2 Current understanding of invasive African *Salmonella* virulence

Genome-based studies of African *Salmonella* have revealed an accumulation of pseudogenes in pathways associated with intestinal colonisation, similar to host-restricted *Salmonella* serovars such as *S.* Typhi, suggesting that African *Salmonella* may be adapting towards an extra-intestinal lifestyle via genome degradation (Feasey *et al.*, 2016; Kingsley *et al.*, 2009; Nuccio and Bäumler, 2014; Okoro *et al.*, 2015; Pulford *et al.*, 2020). Elsewhere, various phenotypic studies have attempted to identify the virulence traits or factors important for the establishment of systemic disease by African *Salmonella* (**Table 1.2**). In African *S.* Typhimurium ST313, the outer membrane protease PgtE is produced at higher levels, conferring increased resistance to serum-mediated killing (Hammarlöf *et al.*, 2018). ST313 also does not

express the SPI-2-encoded effector protein SseI, which enables increased dissemination of ST313 via infected migratory dendritic cells (Carden *et al.*, 2017). Additional reports have shown that African S. Typhimurium ST313 displays higher macrophage survival/replication and invasiveness, and lower cytotoxicity compared to non-ST313 strains in cell and animal infection models, and ST313 has reduced expression of genes responsible for pro-inflammatory cytokine production and inflammasome activation (e.g. flagella) (Carden *et al.*, 2015; Parsons *et al.*, 2013; Ramachandran *et al.*, 2015; Yang *et al.*, 2015).

Much less is known about African *S.* Enteritidis. Feasey *et al.* (2016) was the first study to report a virulence phenotype for African *S.* Enteritidis, specifically that African *S.* Enteritidis was less capable of colonisation of systemic sites such as liver and spleen than gastroenteritis-associated *S.* Enteritidis in an avian infection model; this contrasts with observations in African *S.* Typhimurium ST313 (Parsons *et al.*, 2013). Recently, MacKenzie *et al.* (2019) investigated the biofilm formation capabilities of African *S.* Enteritidis, and showed that the inability of African *S.* Enteritidis to form the RDAR (red, dry and rough) morphotype can be attributed to key SNPs in the promoter region of the *csgD* gene, which controls RDAR expression. Biofilm formation has been proposed to aid in the survival and persistence of *Salmonella* in the environment, and the lack of biofilm formation could be seen as an adaptation towards human-to-human transmission, as observed in host-restricted serovars such as *S.* Typhi (Devaraj *et al.*, 2021; Harrell *et al.*, 2021). Clearly, we are only just beginning to understand the virulence of African *Salmonella* (in particular *S.* Enteritidis). Elucidating the molecular mechanisms used by African invasive *S.* Enteritidis to cause disease is an area of much-needed research.

Table 1.2 Phenotypic virulence studies of African *S.* Typhimurium and *S.* Enteritidis. Adapted and extended from Owen (2017)

Study	Key strains	Virulence phenotypes described
Studies on African *S.* Typhimurium		
Parsons *et al.* (2013)	• D23580 (ST313) • Q456 (ST313) • 4/74 (ST19) • F98 (ST19)	• ST313 is capable of causing invasive disease in chickens • Compared to ST19, ST313 causes lower intestinal colonisation and greater invasiveness (spread to systemic sites)
Goh *et al.* (2013)	• D23580 (ST313) • LT2 (ST19) • CVD1901 (Paratyphi A)	• A higher amount of complement is required for antibody-mediated serum killing of D23580, compared to LT2 and CVD1901
Herrero-Fresno *et al.* (2014)	• 02-03/002 (ST313) • 4/74 (ST19) • 14028 (ST19)	• ST313 possess the *st313-td* (*bstA*) gene, which increases replication in murine macrophages (J774) and increases invasiveness in mice (C57/BL6) relative to ST19, but does not affect invasion of epithelial cells (HeLa)
Yang *et al.* (2015)	• D23580 (ST313) • A130 (ST313) • SL1344 (ST19) • *S.* Typhi Ty2	• ST313 are more acid resistant, more motile, and more invasive in BALB/c mice than ST19
Carden *et al.* (2015)	• D23580 (ST313) • A130 (ST313) • SL1344 (ST19) • DT104 (ST19)	• ST313 invades non-phagocytic cells less efficiently and induces less inflammasome activation than ST19 isolates • Expression of *sopE2* and *fliC* is lower in ST313 than in ST19
Ramachandran *et al.* (2015)	• D23580 (ST313) • SL1344 (ST19) • Malian ST313 and ST19 isolates • *S.* Typhi Ty2 and *S.* Paratyphi A ATCC9150	• ST313 were phagocytosed more efficiently and showed higher levels of survival and replication in cultured and primary murine and human macrophages compared to ST19, but at lower levels compared to typhoidal strains • ST313 induced less apoptosis in infected macrophages, lower levels of proinflammatory cytokines and have reduced flagellin production
Singletary *et al.* (2016)	• D23580 (ST313) • 14028 (ST19)	• Loss of stationary phase KatE, which is protective in high density bacterial cultures
Carden *et al.* (2017)	• D23580 (ST313) • SL1344 (ST19)	• *sseI* gene is pseudogenised in ST313, facilitating hyperdissemination from the gut to systemic sites via migratory DCs in the streptomycin pre-treated mouse model (C57BL/6)
Hammarlöf *et al.* (2018)	• 4/74 (ST19) • D23580 (ST313)	• D23580 contains a SNP in the promoter of the *pgtE* gene, resulting in higher levels of the outer membrane protease PgtE which confers increased resistance to serum-mediated killing

Table 1.2 *(continued)* Phenotypic virulence studies of African *S.* Typhimurium and *S.* Enteritidis. Adapted and extended from Owen (2017)

Study	Key strains	Virulence phenotypes described
***(continued)* Studies on African *S.* Typhimurium**		
Honeycutt *et al.* (2020)	• 4/74 (ST19) • D23580 (ST313)	• D23580 contains two SNPs in the *macAB* genes that inactivates the MacAB-TolB channel • D23580 *macAB* variant does not contribute to its enhanced intra-macrophage replication phenotype but contributes to systemic infection in *Nramp1* negative mice, which are more permissive for *Salmonella* infections
Preciado-Llanes *et al.* (2020)	• African ST313 lineage I and lineage II isolates (including D23580) • UK ST313 isolates • 4/74 and LT2 (ST19) • *S.* Typhi and *S.* Paratyphi	• African ST313 lineage II isolates evade mucosal-associated invariant T (MAIT) cells activation by over-expressing the RibB enzyme involved in the riboflavin pathway
Lê-Bury *et al.* (2020)	• D23580 (ST313) • 4/74 (ST19)	• D23580 survives better in HIV-infected primary human macrophages than 4/74
Sokaribo *et al.* (2021)	• D23580 (ST313) • 14028 (ST19)	• D23580 contains a SNP in *STM1987* that results in reduced cellulose production, increased survival inside macrophages and increased *in vivo* fitness
Studies on African *S.* Enteritidis		
Feasey *et al.* (2016)	• D7795 • A1636 (Global Epidemic isolate from Africa)	• D7795 is less invasive than A1636 in an avian model of infection
MacKenzie *et al.* (2019)	• D7795	• D7795 does not form the RDAR morphotype, due to SNPs in the promoter region of the *csgD* gene that encodes a Response Regulator protein

1.6 An introduction to transposon insertion sequencing

Transposons are mobile genetic elements that can move within or between genomes, mediated by the action of transposase enzymes (van Opijnen and Camilli, 2013). Since their discovery, transposons have become important tools in molecular biology, as they can be used to generate large number of transposon mutants in an organism of interest to facilitate study of gene function. Early analytic methods such as genetic footprinting and signature-tagged mutagenesis (STM) combined transposon mutagenesis with polymerase chain reaction (PCR) to determine the location of the transposon insertion (Hensel *et al.*, 1995; Smith *et al.*, 1995). Specifically, STM utilised transposon-containing unique sequences (signature tags) to identify individual mutants within a pool of 1510 mutants, allowing for parallel analysis of mutant strains (Hensel *et al.*, 1995). Subsequent methods such as transposon site hybridisation (TraSH) and transposon-mediated differential hybridisation (TMDH) employed DNA microarray technology to scale up the number of mutants that could be analysed simultaneously (Chaudhuri *et al.*, 2009a; Sassetti *et al.*, 2001). The advent of next-generation sequencing (NGS) technologies facilitated the development of transposon insertion sequencing (TIS) methods. Interestingly, the first TIS study was done in 1999 on *Mycobacterium genitalium*, although the high costs of sequencing limited its widespread application at that time (Hutchison *et al.*, 1999). Four variations of TIS methods were published in 2009, namely Insertion sequencing (INSeq), high-throughput insertion tracking by deep sequencing (HITS), transposon-directed insertion site sequencing (TraDIS), and transposon sequencing (Tn-Seq) (reviewed in van Opijnen and Camilli, 2013; also see **Table 1.3**). TIS methods have since been extended to incorporate other technologies such as density gradient centrifugation, cell sorting and single-cell methods (reviewed in Cain *et al.*, 2020).

Table 1.3 Key publications describing applications of transposon mutagenesis for the functional characterisation of bacterial genomes[a]

Study	Organism	Transposon	Application(s)	Technique
Hensel *et al.* (1995)	*S*. Typhimurium	Tn*5*	Genes required for virulence in murine model of typhoid fever	Signature-tagged mutagenesis (STM)
Sassetti *et al.* (2001)	*Mycobacterium bovis* bacillus Calmette-Guérin	Mariner	Genes required for growth in minimal medium	Transposon site hybridization (TraSH)
Chaudhuri *et al.* (2009a)	*Staphylococcus aureus*	Mariner	Essential genes	Transposon mediated differential hybridisation (TMDH)
Chaudhuri *et al.* (2009b)	*S.* Typhimurium	Tn*5*, Mu	Genes required for infection of mice	TMDH
Goodman *et al.* (2009)	*Bacteroides thetaiotaomicron*	Mariner	Genes required for colonisation of the human gut	Insertion sequencing (INSeq)
Gawronski *et al.* (2009)	*Haemophilus influenzae*	Mariner	Genes required for persistence in the murine lung	High-throughput insertion tracking by deep sequencing (HITS)
Langridge *et al.* (2009)	*S.* Typhi	Tn*5*	Genes (1) required for growth in rich medium; (2) advantageous or disadvantageous for growth in rich medium; and (3) required for bile tolerance	Transposon-directed insertion site sequencing (TraDIS)
van Opijnen *et al.* (2009)	*Streptococcus pneumoniae*	Mariner	Essential genes; genetic interactions between genes of interest	Transposon sequencing (Tn-Seq)

[a] Additional publications are cited in Cain *et al.* (2020)

A typical TIS workflow begins with the construction of a transposon mutant library, ideally with high insertion densities (i.e. highly saturated) such that each gene locus contains multiple unique insertions (Cain *et al.*, 2020; Chao *et al.*, 2016). Genomic DNA is extracted from the mutant library pool, and the transposon flanking regions are sequenced to determine the position and frequency of transposon insertion. Sequencing the initial library will reveal genes that lack insertions; while some studies refer to such genes as essential genes (Rancati *et al.*, 2018), others use the term "required" to reflect the fact that "essentiality" is always relative to the growth condition (Barquist *et al.*, 2013a). This definition of the term "required" has been adopted in this thesis. Non-essential genes for a particular growth condition are dispensable and contain insertions (Canals *et al.*, 2019a).

The transposon library can be also subjected to *in vitro* or *in vivo* experimental conditions. By comparing the relative frequency of each mutant in the population before (i.e. input) and after (i.e. output) experimental selection, genomic features that show a decrease in insertion frequency in the output are likely to be important for survival and/or growth (i.e. fitness), while features where insertions show an increase in frequency in the output can be assumed to exert a disadvantageous effect to the organism under normal growth conditions (Cain *et al.*, 2020).

Transposon mutagenesis has revealed many important *Salmonella* virulence functions. The first STM study of *Salmonella* genes focused on genes required for mice infection and led to the identification of SPI-2 (Hensel *et al.*, 1995). Subsequently, various transposon mutagenesis-based screens have significantly improved our understanding of *Salmonella* gene function (**Table 1.4**).

Table 1.4 Transposon mutagenesis-based screens to study *Salmonella* gene function

Organism	Library	Experimental conditions	Reference
S. Typhi WT26 pHCM1	• ~1.1 million individual mutants • Between 200,000 and 300,000 individual insertion sites • Average one insertion site for every 15 – 20 bp, >80 inserts per gene	• Growth under standard laboratory conditions (6 passages in LB) and biologically relevant conditions (LB supplemented with aro mix and Oxgall to test for bile tolerance)	Langridge *et al.* (2009)
S. Enteritidis G1 Nal^R	• Mini-Tn*5* mutant libraries • 4,992 mutants	• Caco2 and LMH (chicken intestinal cells) invasion • Survival in HD-11 chicken macrophages • Growth in egg albumen	Shah *et al.* (2012)
S. Enteritidis P125109	• Mini-Tn*5* mutant libraries • ~54,000 mutants	• Intraperitoneal infection of BALB/c mice	Silva *et al.* (2012)
S. Typhimurium 4/74 (and SL1344)	• 5184 Mu and 5184 Mini-Tn*5* mutants (mice) (from previous TMDH study) • 8550 mini-Tn5 mutants (chickens, pigs, calves)	• Intravenous infection of BALB/c mice • Oral infection of chickens, pigs and calves – Pools of 475 mutants screened in pigs and calves – Pools of 95 mutants screened in chickens	Chaudhuri *et al.* (2013)
S. Typhimurium SL1344	• Mu and Tn*5* mutant libraries • Screened 21 pools of 480 Tn mutants in each pool, covering ~10000 Tn*5* and Mu mutants	• Intravenous infection of C57BL/6 wild-type and immunodeficient *gp91$^{-/-}$ phox* mice	Grant *et al.* (2016)
S. Typhimurium 14028 NA^R	• Two libraries each containing approximately 325,000 mutants • Constructed by biparental mating using *Escherichia coli* SM10 *λpir* carrying a pBAM1 transposon-delivery plasmid vector as the donor strain	• Growth in presence of different concentrations of iron chelator Dipyridyl	Karash and Kwon (2018)
S. Typhi WT26 pHCM1	• Tn*5* insertion library with ~250,000 unique insertion sites	• Survival in water	Kingsley *et al.* (2018)
S. Typhimurium D23580	• Tn*5* library with at least 500,000 unique insertion sites	• Macrophage infection • *in vitro* growth in LB and InSPI-2 media	Canals *et al.* (2019a)
S. Enteritidis P125109, *S.* Typhimurium 14028, *S.* Newport C4.2	• Tn*5* libraries	• Survival on low moisture foods	Jayeola *et al.* (2020)

1.7 Research aims and objectives

S. Enteritidis is among the most commonly isolated serotypes responsible for NTS infections worldwide, and the second most common cause of invasive bloodstream infections in sub-Saharan Africa (Section 1.5). Despite this, the vast majority of *Salmonella* virulence studies have focused on *S.* Typhimurium as the model organism. A search for the term "Typhimurium virulence" or "Enteritidis virulence" from the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) on 29th April 2021 identified 5,396 and 1,174 publications, respectively. While various reports have revealed many similarities in virulence strategies used by both serovars (Section 1.4), there are also serovar-specific differences, exemplified by the Silva *et al.* (2012) study describing *S.* Enteritidis-specific genes involved in murine infection. Furthermore, research into African *S.* Enteritidis is clearly lacking (Section 1.5.2).

The overall aim of this PhD project is to advance our understanding of gene function in African *S*. Enteritidis by exploring the genes that contribute to fitness during *in vitro* growth and for intracellular survival and replication in macrophages. Experiments were performed in parallel on representative strains from both Global Epidemic and African *S*. Enteritidis clades. By comparing the findings between the two *S.* Enteritidis clades, it is hoped that genes that display a differential pattern between the two variants of *S*. Enteritidis associated with different disease syndromes (gastroenteritis vs. bloodstream infection) will be identified, and shed light on the molecular basis of the difference in infection phenotypes. The findings can also be compared more broadly against existing African *S.* Typhimurium datasets, to search for similarities between the two African strains associated with iNTS disease. These findings could identify common virulence determinants, as well as extend our current understanding of serovar-specific requirements for *Salmonella* infection.

At the beginning of the PhD project, the Hinton Laboratory had just completed a large-scale transcriptomic analysis of the representative African *S.* Typhimurium strain D23580 in 16 infection-relevant conditions and within murine macrophages (Canals *et al.*, 2019b). An initial goal of the PhD project was to perform a similar transcriptomic study of Global Epidemic and African *S.* Enteritidis during murine macrophage infection, to identify genes that are differentially-expressed between the two strains. This RNA-seq-based investigation necessitated the isolation of *Salmonella* RNA from infected macrophages using our published differential-lysis approach (Eriksson *et al.*, 2003; Hautefort *et al.*, 2008; Srikumar *et al.*, 2015). This was the motivation behind Chapter 3, where I describe experiments to optimise the existing protocol for the isolation of *Salmonella* RNA from infected macrophages, intended for use with *S.* Enteritidis macrophage infection experiments.

Following two years on protocol optimisation, the technical challenges with intra-macrophage *Salmonella* RNA isolation remained unresolved. TIS was then used to assay *S.* Enteritidis gene requirements for *in vitro* growth (Chapter 4) and fitness during macrophage infection (Chapter 5). Coincidentally, the Hinton Laboratory published a TIS-based study of African *S.* Typhimurium D23580 in *in vitro* growth and macrophage infection around the same time (Canals *et al.*, 2019a), providing relevant datasets against which the *S.* Enteritidis datasets could be compared.

Specifically, the objectives of this PhD project are to:

- Optimise the protocol for isolation of *Salmonella* RNA from infected macrophages (Chapter 3);
- Construct transposon mutant libraries of representative Global Epidemic *S.* Enteritidis strain P125109 and African *S.* Enteritidis strain D7795;

- Assess the biological contribution of genes in P125109 and D7795 by TIS during *in vitro* growth (Chapter 4) and infection of macrophages (Chapter 5); and
- Perform inter-strain and inter-serovar comparisons between the *S.* Enteritidis and representative African *S.* Typhimurium strain D23580 TIS datasets.

Chapter 2 :

Materials and Methods

2.1 Chemicals and reagents

All chemicals and reagents used in this thesis, along with purchase supplier information, are listed in **Table 2.1**.

2.2 Media and antibiotics

Recipes for all media used are shown in **Table 2.1**. Unless otherwise stated, all media and media supplements were made with ultrapure type I water (MilliQ H_2O) with a resistivity of 18.2 MΩ.cm at 25°C, produced by the PURELAB flex 2 system (ELGA LabWater). Lysogeny broth, Lennox formulation (LB media), Terrific broth (TB) and SOC media (excluding the glucose component) were sterilised by autoclaving using a standard sterilisation cycle of 121°C at 15 psi (100 kPa) above atmospheric pressure for 15 min. InSPI2 and NonSPI2 media are phosphate-carbon-nitrogen (PCN)-based synthetic minimal media composed of multiple ingredients (Löber *et al.*, 2006). To prepare InSPI2 and NonSPI2 media, the ingredients were prepared and sterilised separately either by autoclaving or syringe-driven using Millex-GP 0.22 µm (hydrophilic Polyethersulfone [PES] 33 mm) filter units, then combined and sterilised once more by vacuum-driven filtration through 0.22 µm Steritop™ filter unit (Merck Millipore). Glucose solutions used in growth media were sterilised by syringe-driven filtration. Cell culture media and media supplements (for experiments described in Section 2.5) and reagents for RNA experiments (described in Section 2.6) were purchased as ready-to-use formulations.

LB agar was prepared by the addition of 15 g/L Bacto Agar to LB media prior to autoclaving. Molten agar was cooled to 50°C before aliquoting 25 mL into sterile Petri dishes. Lids were left ajar under sterile conditions to allow the plates to cool and dry for 30 min. Agar plates were stored in sealed plastic bags at 4°C and brought to room temperature before use.

Antibiotic stock solutions were made in MilliQ H_2O and sterilised by syringe-driven filtration through a 0.22 µM filter, or in 100% methanol, as specified in **Table 2.1**. Aliquots were stored at −20°C and thawed on ice before use. Where agar was supplemented with antibiotics, stock solutions were added to sterile molten agar at 50°C and mixed thoroughly before pouring the plates.

Table 2.1 Chemicals and reagents used in this study

Use	Chemical / Reagent	Supplier	Catalogue Number	Stock concentration	Final concentration
For bacterial culture					
LB media (Lennox)	Tryptone	Appleton Woods	MN649	N/A	10 g/L
	Bacto-Yeast Extract	Appleton Woods	DM832	N/A	5 g/L
	NaCl	Sigma-Aldrich	S3014	N/A	5 g/L
	Bacto-Agar	Appleton Woods	214010	N/A	15 g/L
Terrific broth	Terrific broth, modified (containing 12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4)	Sigma-Aldrich	T0918	N/A	47.6 g/L
	Glycerol	Sigma-Aldrich	49767	N/A	8 mL/L
InSPI2 media	MES (pH 5.8)	Sigma-Aldrich	M8250	400 mM	80 mM
	Tricine	Sigma-Aldrich	T5817	400 mM	4 mM
	$FeCl_3$	Sigma-Aldrich	236489	100 mM	100 µM
	K_2SO_4	Sigma-Aldrich	P0772	376 mM	376 µM
	NaCl	Sigma-Aldrich	S3014	2.5 M	50 mM
	K_2HPO_4/KH_2PO_4 (pH 5.8)[#]	N/A	N/A	100 mM	0.4 mM
	Glucose	Sigma-Aldrich	G8270	20% (w/v)	0.4%
	NH_4Cl	Sigma-Aldrich	A9434	1.5 M	15 mM
	$MgSO_4$	Sigma-Aldrich	83266	1 M	1 mM
	$CaCl_2$	Sigma-Aldrich	C3306	1 M	0.01 mM
	Micronutrients solution^	N/A	N/A	10,000X	1X

Table 2.1 *(continued)* Chemicals and reagents used in this study

Use	Chemical / Reagent	Supplier	Catalogue Number	Stock concentration	Final concentration
***(continued)* For bacterial culture**					
NonSPI2 media	MOPS (pH 7.4)	Sigma-Aldrich	M1254	400 mM	80 mM
	Tricine	Sigma-Aldrich	T5817	400 mM	4 mM
	$FeCl_3$	Sigma-Aldrich	236489	100 mM	100 µM
	K_2SO_4	Sigma-Aldrich	P0772	376 mM	376 µM
	NaCl	Sigma-Aldrich	S3014	2.5 M	50 mM
	K_2HPO_4/KH_2PO_4 (pH 7.4)[#]	N/A	N/A	100 mM	25 mM
	Glucose	Sigma-Aldrich	G8270	20% (w/v)	0.4%
	NH_4Cl	Sigma-Aldrich	A9434	1.5 M	15 mM
	$MgSO_4$	Sigma-Aldrich	83266	1 M	1 mM
	$CaCl_2$	Sigma-Aldrich	C3306	1 M	0.01 mM
	Micronutrients solution^	N/A	N/A	10,000X	1X
[#]K_2HPO_4/KH_2PO_4 (pH 5.8 or pH 7.4)	K_2HPO_4	Sigma-Aldrich	60353	1 M	Mixed in appropriate volumes to give respective pH
	KH_2PO_4	Sigma-Aldrich	P8416	1 M	
^Micronutrients solution	Na_2MoO_4	Sigma-Aldrich	737860	100 µM	10 nM
	Na_2SeO_3	Sigma-Aldrich	S5261	100 µM	10 nM
	H_3BO_3	Sigma-Aldrich	B6768	40 µM	4 nM
	$CoCl_2$	Sigma-Aldrich	C8661	3 mM	300 nM
	$CuSO_4$	Sigma-Aldrich	C1297	1 mM	100 nM
	$MnCl_2$	Sigma-Aldrich	529680	8 mM	800 nM
	$ZnSO_4$	Sigma-Aldrich	Z4750	10 µM	1 nM

Table 2.1 *(continued)* Chemicals and reagents used in this study

Use	Chemical / Reagent	Supplier	Catalogue Number	Stock concentration	Final concentration
***(continued)* For bacterial culture**					
SOC media	Tryptone	Appleton Woods	MN649	N/A	20 g/L
	Bacto-Yeast Extract	Appleton Woods	DM832	N/A	5 g/L
	NaCl	Sigma-Aldrich	S3014	N/A	0.5 g/L
	KCl	Sigma-Aldrich	P9333	250 mM	2.5 mM
	$MgCl_2$	VWR Chemicals	25108.260	2 M	10 mM
	Glucose	Sigma-Aldrich	G8270	1 M	20 mM
For mammalian cell culture and *in vitro* infection					
General cell culture reagents and supplements	Dulbecco's Modified Eagle Medium, high glucose	Thermo Fisher Scientific	41965-039 / 41965-062	N/A	N/A
	L-glutamine	Thermo Fisher Scientific	25030-024	200 mM	2 mM
	Fetal Bovine Serum, certified, heat inactivated, US origin	Thermo Fisher Scientific	10082-147	N/A	10% (v/v)
	MEM Non-Essential Amino Acids	Thermo Fisher Scientific	11140-035	100X	1X
	Dulbecco's phosphate-buffered saline, calcium, magnesium, glucose, pyruvate	Thermo Fisher Scientific	14287-080	1X	1X
	Recovery™ Cell Culture Freezing Medium	Thermo Fisher Scientific	12648-010	N/A	N/A
	Trypan Blue, 0.4%	Thermo Fisher Scientific	15250-061	0.4%	0.2%

Table 2.1 *(continued)* Chemicals and reagents used in this study

Use	Chemical / Reagent	Supplier	Catalogue Number	Stock concentration	Final concentration
***(continued)* For mammalian cell culture and *in vitro* infection**					
Infection protocol reagents	Serum from male BALB/cAnNCrl mice	Charles River	N/A	N/A	10% (v/v)
	Gentamicin solution	Thermo Fisher Scientific	15710-049	10 mg/mL	Various concentrations
For RNA isolation					
Ready-to-use reagents	Chloroform	Sigma-Aldrich	C2432	N/A	N/A
	Isopropanol	Sigma-Aldrich	19516	N/A	N/A
	Ethanol	Sigma-Aldrich	E7023	N/A	Various concentrations
	Phenol (pH 4.3)	Sigma-Aldrich	P4682	N/A	1%
	TRIzol®	Fisher Scientific	12044977	N/A	N/A
	TRIzol® Max™ Bacterial RNA Isolation Kit	Fisher Scientific	10339372	N/A	N/A
	RNAzol RT	Sigma-Aldrich	R4533	N/A	N/A
	DEPC-treated water	Fisher Scientific	10514065	N/A	N/A
	SDS, 20% solution	Fisher Scientific	10625405	20%	0.2%
Acidic phenol-chloroform extraction	Trizma® base	Sigma-Aldrich	T1503	1 M	10 mM
	EDTA	Sigma-Aldrich	E4884	0.5 M	1 mM
	Sodium acetate	Sigma-Aldrich	S8625	3 M	0.3 M
	Lysozyme	Sigma-Aldrich	L6876	50 mg/mL	0.5 mg/mL
	Proteinase K	Zymo Research	D3001-2-20	20 mg/mL	100 µg/mL

Table 2.1 *(continued)* Chemicals and reagents used in this study

Use	Chemical / Reagent	Supplier	Catalogue Number	Stock concentration	Final concentration
***(continued)* For RNA isolation**					
Differential centrifugation	Percoll™	Fisher Scientific	10607095	N/A	N/A
	Percoll™ PLUS	Fisher Scientific	11500744	N/A	N/A
	Sucrose	Fisher Scientific	10634932	N/A	Various concentrations
Miscellaneous					
Media supplement	Kanamycin monosulphate	Melford	K22000	50 mg/mL	50 µg/mL
	Tetracycline	Sigma-Aldrich	87128	25 mg/mL (methanol)	25 µg/mL
	Gentamicin sulphate	Melford	G38000	40 mg/mL	20 µg/mL
	L-arabinose	Sigma-Aldrich	A3256	20% (w/v)	0.20%
General reagent	Phosphate buffered saline	Fisher Scientific	11503387	1X	1X

2.3 General microbiological techniques

2.3.1 Bacterial strains and growth conditions

Bacterial strains used in this thesis are listed in **Table 2.2**. Bacteria were routinely grown in a volume of 5 mL LB broth inoculated from single colonies on LB plates for 16 h at 37°C with shaking at 220 rpm (this procedure is referred to as 'overnight culture' unless otherwise described). For experiments, overnight cultures were used to sub-inoculate 1:1000 in 25 mL LB broth in 250 mL sterilised Erlenmeyer flasks and grown at 37°C with 220 rpm agitation until the desired optical density (OD). For growth in minimal media (InSPI2 and NonSPI2), 1 mL of overnight culture was harvested by centrifugation at 12,000 x *g* at room temperature for 1 min. Cell pellets were washed three times in phosphate buffered saline (PBS) before sub-inoculation. Where necessary, antibiotics were used at the final concentrations listed in **Table 2.1.**

Bacterial strains were maintained as frozen stocks at −80°C for the duration of this project. To prepare frozen stocks, 900 µL of overnight bacterial cultures were mixed with 600 µL autoclaved 50% (v/v) glycerol in a 1.8 mL cryogenic vial (STARLAB). Bacterial colonies were maintained on agar plates at 4°C for a maximum of one week.

OD measurements were taken using a Jenway 67-series spectrophotometer at a wavelength of 600 nm. Bacteria numbers were determined using colony forming unit (CFU) counts. 10-fold serial dilutions of cultures (up to a factor of 10^{-8}) were carried out in sterile PBS and triplicate 20 µL drops plated on LB agar plates (Miles and Misra method).

Table 2.2 Bacterial strains used in this study

Name	Description[a]	Source / Reference
***S.* Typhimurium strains and derivatives**		
4/74	*S.* Typhimurium 4/74; reference ST19 strain isolated from bovine infection	Rankin and Taylor (1966)
4/74 Δ*phoPQ*	4/74 *phoPQ* deletion mutant constructed using λ Red recombination method	Colgan *et al.* (2016)
4/74 *rpsM::gfp*+	4/74 carrying a chromosomal *rpsM::gfp* fusion linked to the *cat* gene (for chloramphenicol resistance); constructed by P22 phage transduction from a SL1344 strain carrying a chromosomal *rpsM::gfp* fusion into wild-type 4/74 background; Cm^R	Hautefort *et al.* (2003)
***S.* Enteritidis strains and derivatives**		
P125109	Wild-type; reference strain isolated from an outbreak of human food poisoning in the United Kingdom; belongs to Global Epidemic clade as described in Feasey *et al.* (2016)	Thomson *et al.* (2008)
P125109 Δ*phoPQ*	P125109 *phoPQ* deletion mutant constructed using λ Red recombination method	This study
D7795	Wild-type; reference invasive isolate from the blood of a Malawian child in 2000; belongs to Central/Eastern African clade as described in Feasey *et al.* (2016)	Feasey *et al.* (2016)
D7795 Δ*phoPQ*	D7795 *phoPQ* deletion mutant constructed using λ Red recombination method	This study
A1636	Wild-type; bloodstream isolate from Malawi; belongs to Global Epidemic clade as described in Feasey *et al.* (2016)	Feasey *et al.* (2016)
CP255	Wild-type; bloodstream isolate from Democratic Republic of Congo; belongs to Central/Eastern African clade as described in Feasey *et al.* (2016)	C. Parry; Perez-Sepulveda *et al.* (2021)

[a] Relevant antibiotic resistances are indicated by ^{R}: Cm, chloramphenicol

2.3.2 Growth curves

Growth curves were routinely performed in 96-well microtitre plates (Greiner, product code 655161). To standardise inoculum size, OD_{600} of overnight cultures were adjusted to OD_{600} 1.0 before inoculating the wells of a 96-well microtitre plate, each containing 200 µL of media, at a 1:100 dilution. The plate was covered with a plastic lid (Greiner, product code 656171) and incubated at 37°C with shaking at 900 rpm in a FLUOstar Omega microplate reader, with OD_{600} readings taken every 30 min for 18 h. Triplicate wells were used for each strain, and wells containing non-inoculated media were included as negative controls.

ESP is the well-defined "early stationary phase" growth condition that has been used extensively to study *Salmonella* biology (e.g. Kröger *et al.*, 2013; Pfeiffer *et al.*, 2007). Most experiments in this study that involved growing *Salmonella* cultures in LB to ESP referred to growing the bacterial cultures at 37°C with shaking until OD_{600} reached 2.0. In Section 2.7.2, *S.* Enteritidis P125109 and D7795 transposon mutant libraries were passaged in LB, NonSPI2 or InSPI2 at ESP, which has to be defined differently due to the different growth rates between the two strains in the three media. Growth curves were performed by growing the wild-type strain in the respective media in a culture volume of 25 mL inoculated to a starting OD_{600} of 0.05. For growth curves in LB, OD_{600} was measured at 1-h intervals over 8 h. For P125109 growth curves in NonSPI2 and InSPI2, OD_{600} was measured at 2-h intervals starting at about 4 h, over a 14-h period. For D7795 growth curves in NonSPI2 and InSPI2, OD_{600} was measured at 4-h intervals over a 28-h period. OD_{600} measurements were taken using a Jenway 67-series spectrophotometer.

2.4 Molecular genetic techniques

2.4.1 Plasmids and primers

All plasmids and primers used in this thesis are listed in **Table 2.3** and **Table 2.4** respectively. Plasmids were isolated from *Escherichia coli* (*E. coli*) strains using the ISOLATE II Plasmid Mini Kit (Bioline) following manufacturer's instructions, except column elution was always carried out in molecular grade H_2O. Primers for construction of deletion mutants of *Salmonella* were purchased from MWG Eurofins (Germany) and were HPSF (High-Purity, Salt-Free)-purified. Primers used in the next-generation sequencing of *Salmonella* transposon mutant libraries were purchased from Integrated DNA Technologies (USA) and were HPLC (high performance liquid chromatography)-purified.

Table 2.3 Plasmids used in this study

Name	Description[a]	Source / Reference
pKD4	Template plasmid for gene deletion; Amp^R, FRT-flanked *kan* resistance cassette	Datsenko and Wanner (2000)
pSIM5-*tet*	λ Red recombination plasmid, thermo-inducible; Tet^R	Koskiniemi *et al.* (2011)
pKD46-*aacC1*	λ Red recombination plasmid, arabinose-inducible; Gm^R (modified from original pKD46 Amp^R plasmid by N. Wenner)	Datsenko and Wanner (2000)
pCP20-*tet*	Plasmid carrying FLP recombinase to remove kanamycin resistance from pKD4 derived resistance cassette insertions; Tet^R	Kintz *et al.* (2015)
pCP20-Gm	Plasmid carrying FLP recombinase to remove kanamycin resistance from pKD4 derived resistance cassette insertions; Gm^R	Doublet *et al.* (2008)

[a] Relevant antibiotic resistances are indicated by ^{R}: Amp, ampicillin; Gm, gentamicin; Km, kanamycin; Tet, tetracycline.

Table 2.4 Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
For construction of deletion mutants in *Salmonella*			
pKD4_F	GTGTAGGCTGGAGCTGCTTC	For amplification of *kan* resistance cassette from pKD4. Attached to each upstream gene specific primer	Colgan (2015)
pKD4_R	CATATGAATATCCTCCTTAG	For amplification of *kan* resistance cassette from pKD4. Attached to each downstream gene specific primer	Colgan (2015)
phoQ_KO_F	CGATTATAACGGATGCTTAACGAGATGCGTGGAAGAACGCACAGAAATGT	Binds outside *phoQ* CDS for removal of *phoP* and *phoQ* genes	Colgan (2015)
phoPQ_KO_R	CACCATAATCAACGCTAGACTGTTCTTATTGTTAACACAAGGGAGAAGAG	Binds outside *phoP* start codon for removal of *phoP* and *phoQ* genes	Colgan (2015)
Fw-phoPQ_ext	GTGGTTTACCGCCTGTACC	External primer for checking deletion of *phoPQ* following FRT recombination	R. Canals
Rv-phoPQ_ext	GACGCCGGCAAATTATATC	External primer for checking deletion of *phoPQ* following FRT recombination	R. Canals
For next-generation sequencing of *Salmonella* transposon mutant libraries			
PE PCR Primer 2.0	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T	Universal Illumina reverse primer	Illumina
PE PCR Tn-1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**CGTGAT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CGTGAT**	Canals *et al.* (2019a)
PE PCR Tn-2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**ACATCG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ACATCG**	R. Canals
PE PCR Tn-3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**GCCTAA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCCTAA**	R. Canals

Table 2.4 *(continued)* Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
	***(continued)* For next-generation sequencing of *Salmonella* transposon mutant libraries**		
PE PCR Tn-4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TGGTCA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TGGTCA**	Canals *et al.* (2019a)
PE PCR Tn-5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CACTGT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CACTGT**	Canals *et al.* (2019a)
PE PCR Tn-6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**ATTGGC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ATTGGC**	R. Canals
PE PCR Tn-7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GATCTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GATCTG**	Canals *et al.* (2019a)
PE PCR Tn-8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TCAAGT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TCAAGT**	R. Canals
PE PCR Tn-9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CTGATC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CTGATC**	R. Canals
PE PCR Tn-10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**AAGCTA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **AAGCTA**	Canals *et al.* (2019a)
PE PCR Tn-11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GTAGCC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GTAGCC**	R. Canals
PE PCR Tn-12	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TACAAG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TACAAG**	Canals *et al.* (2019a)

Table 2.4 *(continued)* Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
***(continued)* For next-generation sequencing of *Salmonella* transposon mutant libraries**			
PE PCR Tn-13	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TTGACT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TTGACT**	This study
PE PCR Tn-14	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GGAACT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GGAACT**	This study
PE PCR Tn-15	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TGACAT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TGACAT**	This study
PE PCR Tn-16	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GGACGG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GGACGG**	This study
PE PCR Tn-17	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CTCTAC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CTCTAC**	This study
PE PCR Tn-18	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GCGGAC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCGGAC**	This study
PE PCR Tn-19	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TTTCAC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TTTCAC**	This study
PE PCR Tn-20	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GGCCAC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GGCCAC**	This study
PE PCR Tn-21	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CGAAAC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CGAAAC**	This study

Table 2.4 *(continued)* Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
	***(continued)* For next-generation sequencing of *Salmonella* transposon mutant libraries**		
PE PCR Tn-22	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CGTACG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CGTACG**	This study
PE PCR Tn-23	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CCACTC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CCACTC**	This study
PE PCR Tn-24	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GCTACC**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCTACC**	This study
PE PCR Tn-25	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**ATCAGT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ATCAGT**	This study
PE PCR Tn-26	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GCTCAT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCTCAT**	This study
PE PCR Tn-27	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**AGGAAT**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **AGGAAT**	This study
PE PCR Tn-28	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CTTTTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CTTTTG**	This study
PE PCR Tn-29	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TAGTTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TAGTTG**	This study
PE PCR Tn-31	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**ATCGTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ATCGTG**	This study

Table 2.4 *(continued)* Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
***(continued)* For next-generation sequencing of *Salmonella* transposon mutant libraries**			
PE PCR Tn-32	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TGAGTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TGAGTG**	This study
PE PCR Tn-33	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CGCCTG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CGCCTG**	This study
PE PCR Tn-34	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GCCATG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCCATG**	This study
PE PCR Tn-35	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**AAAATG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **AAAATG**	This study
PE PCR Tn-36	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TGTTGG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TGTTGG**	This study
PE PCR Tn-37	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**ATTCCG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ATTCCG**	This study
PE PCR Tn-39	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GTATAG**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GTATAG**	This study
PE PCR Tn-42	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**CGATTA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **CGATTA**	This study
PE PCR Tn-43	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GCTGTA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GCTGTA**	This study

Table 2.4 *(continued)* Primers used in this study

Name	Sequence (5' → 3')	Purpose	Source / Reference
***(continued)* For next-generation sequencing of *Salmonella* transposon mutant libraries**			
PE PCR Tn-44	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**ATTATA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **ATTATA**	This study
PE PCR Tn-45	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**GAATGA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **GAATGA**	This study
PE PCR Tn-46	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT**TCGGGA**GCTTCAGGGTTGAGATGTGT*A	Forward primer for amplification of transposon-flanking region and introduces unique barcode **TCGGGA**	This study

* denotes phosphorothioate bond; XXXXXX is the unique 6-base barcode for multiplexing of samples during Illumina sequencing; underlined text refers to the transposon-specific sequence

2.4.2 Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were carried out in a Mastercycler™ Nexus Thermal Cycler (Eppendorf). For routine amplifications, MyTaq™ Red Mix (Bioline) was used according to the manufacturer's instructions. A standard 50 µL PCR reaction used 100 ng of template DNA and primers at a final concentration of 0.2 µM each. Colony PCR was performed in 20 µL reactions, and primers were added at a final concentration of 0.5 µM each.

Initial denaturation time in the reaction was dependent on the template DNA used. For colony PCR, initial denaturation proceeded for 10 min at 95°C; the time was reduced to 2 min when extracted and purified DNA was used as template. For all PCR reactions, initial denaturation was followed by 30 cycles of denaturation for 15 s at 95°C, annealing for 30 s at the recommended annealing temperature of 55°C (unless otherwise stated), and extension for approximately 30 s per kilobase at 72°C. The cycling was followed by a final extension step at 72°C for 5 min. PCR products were held at 4°C until they were analysed.

2.4.3 Agarose gel electrophoresis

Agarose gels were made using molecular grade Agarose (Bioline). A 50X TAE buffer (approximately pH 8.6) stock solution containing 242 g/L Tris base, 57.1 mL/L acetic acid and 100 mL/L 0.5 M sodium EDTA was used. Gels were made up of TAE buffer (1X, made by diluting 20 mL of 50X stock in 980 mL of deionised H_2O), and typically 1% (w/v) agarose. Midori Green Advanced DNA Stain (NIPPON Genetics EUROPE GmbH) was added to agarose gels to a final concentration of 0.04% (v/v). Agarose gels were typically run at a constant voltage of 90–100 V for 30 min, or as required for sufficient separation of the DNA products. The molecular size of electrophoresed DNA products was estimated by comparison with Hyperladder I (Bioline). Separated DNA was visualised using a UV or blue light illuminator. DNA products were purified

from PCR reactions using the ISOLATE II PCR and Gel Kit (Bioline), following manufacturer's instructions, except column elution was always carried out with molecular grade H_2O.

2.4.4 Preparation of electro-competent cells and electroporation

Overnight cultures were diluted 1:100 into 25 mL LB (without NaCl) and grown at 37°C (or 30°C for strains carrying temperature-sensitive plasmids) at 220 rpm to OD_{600} 0.45 ± 0.05. The appropriate antibiotic was added for plasmid-carrying strains. For strains harbouring the λ Red recombination plasmid pKD46, L-arabinose was added to a final concentration of 0.2%. Cells carrying the pSIM5-*tet* plasmid were grown at 30°C to OD_{600} of 0.40 ± 0.05 and then incubated at 42°C for 15 min with shaking, to stimulate the expression of the λ Red recombinase.

At the desired OD_{600}, bacterial cells were transferred to 50 mL centrifuge tubes, chilled on ice for 10 min, then pelleted by centrifugation at 4°C and 4,000 rpm for 10 min using an Eppendorf 5810R centrifuge. Cells were washed three times in 25 mL ice-cold sterile H_2O, and finally resuspended in 250 µL of ice-cold sterile 10% (v/v) glycerol. Cells were aliquoted into 50 µL volumes for electroporation reactions, or storage at −80°C.

For electroporation, cell aliquots were mixed with 500 ng of target DNA and the suspension was incubated on ice for 5 min before transfer to a pre-chilled 2 mm single-use sterile electroporation cuvette (Geneflow, E6-0060). Electroporation was performed using a 1652076 Gene Pulser Laboratory Benchtop Cell Electroporation Unit (Bio-Rad). A single pulse of 2.5 kV, 25 µF, 200 Ω current was applied to the cuvette, with a time constant between 5 to 6 ms. 1 mL of room temperature LB broth was added to the cuvette, and the cell suspension was transferred to a 1.5 mL microcentrifuge tube and incubated at 37°C for 1 h (or 30°C for 2 h if the cells

contained temperature sensitive plasmids) with shaking at 220 rpm. Afterwards, cells were pelleted by centrifugation at 12,000 x *g* for 2 min, and resuspended in 100 µL sterile LB. The cell suspension was spread onto a LB plate containing the appropriate antibiotic and incubated overnight at 37°C. Cells electroplated without DNA were also plated on the antibiotic agar plate, as a negative control.

2.4.5 Construction of *Salmonella* deletion mutants by λ Red recombineering

phoPQ deletion mutants of *S*. Enteritidis strains P125109 and D7795 were created using the λ Red recombination method (Datsenko and Wanner, 2000). The pKD4 plasmid was used as the template for the constructs used in λ Red recombineering. PCR amplification of the kanamycin resistance cassette flanked by the FRT (FLP recombinase target sequence) sites present in the pKD4 plasmid were carried out under conditions described in Section 2.4.2 (except that annealing temperature was set at 63.7°C, following optimisation with temperature gradient PCR) and primers listed in **Table 2.4**. The expected size of the construct was verified on an agarose gel and the PCR products were purified for use in recombineering. Recombineering procedures were carried out using the λ Red recombination plasmids pSIM5-*tet* in the P121509 background and pKD46-*aacC1* in the D7795 background. Electro-competent cells and electroporation were performed as specified in Section 2.4.4, except that TB was used in the preparation of electro-competent D7795 pKD46-harbouring cells (to maximise bacterial yield and therefore electroporation efficiency). Transformants were selected on LB agar containing 25 µg/mL tetracycline (Tet^{25}) or 20 µg/mL gentamicin (Gm^{20}) at 37°C. Colony PCR was used to confirm the presence of the deletion mutation in transformant colonies.

To generate non-polar, in-frame deletions of *phoPQ*, the corresponding kanamycin resistant derivatives were transformed with the temperature-sensitive pCP20-*tet* (for P125109) or pCP20-Gm (for D7795) plasmid that synthesises the FLP recombinase.

FLP recombinase recognises the FRT sites flanking the kanamycin resistance gene in the recombineering construct, and mediates site-specific recombination between the two FRT sites resulting in excision of the antibiotic resistance gene, leaving behind an 82–85 nucleotide (nt) scar sequence (Datsenko and Wanner, 2000). Electro-competent cells and electroporation were performed as previously described (Section 2.4.4), except that D7795 kanamycin resistant cells were grown in TB. Transformants were selected by overnight growth at 30°C on LB agar plates containing Tet^{25} or Gm^{20}, followed by passaging on LB agar plates at 37°C to cure the pCP20 plasmid. Loss of the antibiotic resistance cassette and pCP20 was confirmed by checking for loss of resistance to kanamycin and tetracycline (or gentamicin). Presence of the gene deletion was also confirmed by colony PCR.

Generally, a P22 bacteriophage transduction step is used to transfer the FRT-mediated gene deletion into a clean wild-type background. However, observations from P22 plaque assays performed with P125109 and D7795 (data not shown) indicated that P22 does not infect these strains readily. To confirm that no mutations were introduced during the recombineering process, one *phoPQ* deletion mutant per strain was whole-genome sequenced (MicrobesNG). Analysis of the sequencing data was performed by Dr Alex Predeus and described in Section 2.8.3.

2.5 General mammalian cell culture and *in vitro* infection methods

2.5.1 Cell culture

The RAW 264.7 (ATCC® TIB-71™) murine macrophage cell line was used for all cell culture experiments in this thesis. RAW 264.7 cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), MEM non-essential amino acids (NEAA) (10% final concentration) and L-glutamine (2 mM final concentration), in vented 75 cm^2 tissue culture flasks (T75; Sarstedt, product code 83.3911.002) at 37°C in the

presence of 5% CO_2. Culture volumes were split and provided with 20 mL of fresh media every 2–3 days (at approximately 80% cell confluence). A single starter culture was maintained until the seventh passage, following which a new batch of cells was started from frozen stocks.

Frozen stocks of RAW 264.7 cells were maintained at −135°C in an ultra-low temperature freezer (Sanyo). To freeze mammalian cells, cells between passages 1 and 3 were pelleted by centrifugation at 1,000 rpm, 5 min, and resuspended in Recovery™ Cell Culture Freezing Medium. The cell suspension was then aliquoted into 1.8 mL cryogenic vials, and frozen overnight at −80°C in a Mr. Frosty™ Freezing Container, before being moved to −135°C storage the following day.

2.5.2 *in vitro* infection of macrophages with *Salmonella*

RAW 264.7 cells, between passages 3 and 7, were used for infection experiments. To prepare sufficient cells, the preceding culture was typically expanded to give 4–6 T75 flasks each containing 20 mL culture volume. Viable cell numbers were determined using a 10 µL aliquot of culture mixed with 10 µL Trypan Blue stain pipetted into a chamber on a Countess Cell Counting Chamber Slide (Thermo Fisher Scientific; for automated counting with Countess II FL Automated Cell Counter) or Improved Neubauer 2 Cell Rhodium Coated Counting Chamber (Hawksley; for manual counting). Visualised under a light microscope, live cells (which exclude Trypan Blue) were counted and the density and therefore total number of viable cells within each flask were estimated. Typically, cells were seeded on tissue culture vessels 24 h prior to infection. 6-well plates (Corning Costar, product code 3516) were used for intra-macrophage replication assays. Tissue culture flasks of 75 cm^2 and 175 cm^2 growth surface area (Sarstedt) were used for macrophage infections intended for intra-macrophage *Salmonella* RNA isolation.

Unless otherwise stated, bacterial overnight cultures for macrophage infection were set up using a single bacterial colony inoculated in 25 mL LB, incubated shaking at 220 rpm and 37°C for 18 h. Inoculum size was standardised by adjusting the OD_{600} of overnight cultures to OD_{600} 2.0, and resuspended in DMEM supplemented with MEM NEAA and L-glutamine. Prior to all macrophage infection experiments, bacteria were opsonised with 10% BALB/c mouse serum in 10 volumes of DMEM for 30 min on ice. Macrophages were infected with *Salmonella* at a Multiplicity of Infection (MOI) of 5–10 (for intra-macrophage replication assays) or 100 (for RNA isolation), and infections were synchronised by 5 min centrifugation at 1,000 rpm (Eppendorf 5810, A-4-81 rotor) at room temperature. This was considered as time 0. After 30 min incubation at 37°C and 5% CO_2, cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and incubated with DMEM + 10% FBS containing 100 µg/mL gentamicin (Gm^{100}) for 1 h to kill extracellular bacteria. For time points beyond 1.5 h post-infection, the cell culture media was replaced with fresh DMEM + 10% FBS containing 10 µg/mL gentamicin (Gm^{10}).

Intracellular bacterial numbers were determined by lysis of infected macrophages at the desired time points with 1% Triton X-100 (in DPBS). For intra-macrophage replication assays, the time points were typically 1.5 h and 15.5 h post-infection. Serial dilutions of the cell lysates were plated onto LB agar plates (containing antibiotics where necessary) and incubated overnight at 37°C for bacterial enumeration.

2.6 RNA isolation and analysis

2.6.1 Isolation of *Salmonella* RNA from bacteria grown *in vitro*

S. Typhimurium strain 4/74 was used for all RNA experiments. Isolation of *Salmonella* RNA from bacteria grown *in vitro* was performed as described in Kröger *et al.* (2013). Briefly, overnight cultures of 4/74 were diluted 1:1000 into 25 mL LB and grown at 37°C, 220 rpm until ESP (OD_{600} = 2.0). 4 mL of *Salmonella* cells were harvested, mixed with 0.4X culture volume of ice-cold 'stop solution' (5% phenol [pH 4.3] 95% ethanol), and incubated on ice for 30 min to prevent RNA degradation. The cells were then pelleted by centrifugation at 4,000 rpm (Eppendorf 5810R, A-4-81 rotor), 4°C for 10 min. The supernatant was discarded, and the cell pellet was resuspended in the residual liquid and transferred into a 1.5 mL microcentrifuge tube. Subsequently, the cell suspension was centrifuged for 1 min at 20,000 × *g* and 4 °C and the supernatant was discarded. The bacterial cell pellet was stored at −80°C until RNA extraction (described in Section 2.6.3).

2.6.2 Isolation of *Salmonella* RNA from infected macrophages

Isolation of *Salmonella* RNA from infected macrophages was based on the protocol developed by Eriksson *et al.* (2003), and performed as described in Srikumar *et al.* (2015) with some modifications. A total of 8 x 10^7 RAW 264.7 macrophage cells were seeded in eight T75 flasks 24 h prior to infection, and infected with bacterial cells at a MOI of 100:1 as described in Section 2.5.2. At 8 h post-infection, infected macrophages were lysed in ice-cold 'RNA stabilisation solution' (containing 0.2% (v/v) SDS, 19% (v/v) ethanol and 1% (v/v) phenol [pH 4.3] in DEPC-treated water) and incubated on ice for 30 min to prevent RNA degradation. Macrophage lysate was transferred to pre-chilled 50 mL centrifuge tubes and centrifuged at 6,000 rpm, 4°C for 30 min to pellet intra-macrophage bacteria. Bacterial pellets were washed three times with ice-cold wash buffer (1% phenol [pH 4.3] 19% ethanol in DEPC-treated water), and finally stored at −80°C until RNA extraction (described in Section 2.6.3).

2.6.3 RNA extraction using TRIzol® reagent

Unless otherwise stated, all RNA extractions were performed using TRIzol (Invitrogen). Bacterial cell pellets (prepared as described in Sections 2.6.1, 2.6.2, 2.6.4 and 2.6.5) were dissolved in 1 mL TRIzol on ice and transferred to a 2 mL heavy phase lock gel tube (5PRIME) into which 400 µL of chloroform was added and immediately mixed for 10 seconds. After incubation at room temperature for 2 min, the mixture was centrifuged at 20,000 × *g* for 15 min. The RNA present in the upper aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube, and precipitated by adding 450 µL of isopropanol and incubated at room temperature for 30 min. The precipitated RNA was then pelleted by centrifugation at 20,000 × *g* for 30 min. The RNA pellet was washed in 350 µL 70% (v/v) ethanol (prepared in DEPC-treated water) and centrifuged at 20,000 × *g* for 10 min. The washed pellet was air-dried, reconstituted in 20 µL (for *Salmonella* grown *in vitro*) or 8 µL (for intra-macrophage *Salmonella*) of DEPC-treated water by shaking (900 rpm) for 5 min in a heating block (65°C) (Peqlab Thriller) and stored at −80°C until analysis.

Eukaryotic control RNA from RAW 264.7 macrophages was extracted using TRIzol, following the manufacturer's recommended protocol for RNA isolation from cells cultured in monolayer. RNA from RAW 264.7 was reconstituted in 50 µL of DEPC-treated water.

2.6.4 Methods to improve separation and recovery of intra-macrophage *Salmonella*

2.6.4.1 Physical capture of bacteria using 0.45 µm filter

Eight T75 flasks of macrophages were infected and lysed as described in Section 2.6.2. Intra-macrophage bacteria were then recovered by a filtration method adapted from Sigal *et al.* (2016). Briefly, the macrophage lysate was centrifuged at 800 x *g*, 4°C for 3 min to pellet unbroken cells and macrophage nuclei. The supernatant was

then vacuum-filtered through a 0.45 µm membrane filter fitted on a sterilised reusable filter unit (Nalgene™). The bacteria-containing membrane filter was transferred to a pre-chilled 50 mL centrifuge tube, and the bacteria were washed off the filter by adding 5 mL of ice-cold wash buffer and vigorous vortexing. The bacterial suspension was aliquoted into 1.5 mL microcentrifuge tubes, and bacteria was pelleted by centrifugation at 20,000 x *g* for 2 min.

2.6.4.2 Coarse filtration with cell strainers

Macrophage lysates prepared from the infection protocol described in Section 2.6.2 were sieved through cell strainers (pluriSelect) of various mesh sizes (1 µm, 5 µm, 10 µm, 15 µm, 20 µm, 30 µm, and 40 µm) to reduce the viscosity of the macrophage lysate. 5 mL of lysate was transferred each time to a cell strainer attached directly to a 50 mL centrifuge tube to allow filtration to occur by gravity alone, or with an additional connector ring fitted with a 20 mL syringe to force low pressure (by pulling the piston of the syringe) to aid the filtration process. Bacteria from the filtrate was then pelleted by differential centrifugation as described in Section 2.6.2.

2.6.4.3 Sucrose cushion centrifugation

A 2.5% (w/v) sucrose cushion (prepared in 1% phenol-19% ethanol solution) was first used to optimise the centrifugation settings. 5 mL cell suspension containing 10^8 ESP 4/74 cells, 10^7 macrophage cells, or macrophage lysate containing bacteria was carefully laid over 2 mL sucrose cushion in a 50 mL centrifuge tube, and centrifuged at 800 x *g*, 1600 x *g*, 2400 x *g*, or 3200 x *g* (Eppendorf 5810R), at 4°C for 5 min. The supernatant and sucrose cushion were discarded, and the pellet was resuspended in 100 µL of PBS (or DPBS for macrophages). A 10 µL drop was visualised on the EVOS FL Cell Imaging System to determine the presence of bacterial or macrophage cells. A similar experiment was then performed to compare 5% (w/v) and 10% (w/v) sucrose

cushions, with 10 mL samples centrifuged through a 5 mL sucrose cushion at 3200 x *g*, at 4°C for 5 min, 15 min and 30 min.

2.6.4.4 Differential centrifugation using microcentrifuge tubes (Eriksson *et al.*, 2003)

Macrophages were infected and lysed as described in Section 2.6.2. Macrophage lysate was aliquoted into 1.5 mL microcentrifuge tubes and centrifuged at 14,000 rpm (Eppendorf 5424R, FA-45-24-11 rotor), 4°C for 10 min to pellet intra-macrophage *Salmonella*.

2.6.4.5 Needle and syringe homogenisation

10^7 uninfected macrophages were lysed using 5 mL SDS-phenol-ethanol lysis solution. A needle with needle gauge of either 21G (outer diameter 0.8 mm x length 40 mm), 23G (0.6 mm x 30 mm), 25G (0.5 mm x 25 mm), 26G (0.45 mm x 16 mm), 27G (0.4 mm x 13 mm) or 30G (0.29 mm x 13 mm), fitted to a 5 mL, 10 mL or 20 mL syringe, was used to draw the lysate through 10 times to homogenise the macrophage lysate. A small volume of lysis solution was drawn up the needle prior to lysate homogenisation. All needles were supplied by BD Microlance.

2.6.4.6 Percoll gradient centrifugation

Percoll (GE Healthcare Life Sciences) gradients were used either as pre-formed gradients, following the protocol of Makinoshima *et al.* (2002), or self-generating gradients, following the general principles described in Lis *et al.* (2014), Tamura *et al.* (1982) and Yuksel *et al.* (2006).

In the Makinoshima *et al.* (2002) protocol, 24 mL of Percoll and 4.5 mL 1% phenol-19% ethanol solution were mixed together in Nalgene™ Oak Ridge High-Speed

PPCO conical bottom centrifuge tubes, then centrifuged at 25,000 x *g* (Sorvall Lynx 4000, Fiberlite F14-14x50cy rotor) for 60 min at 4°C to generate a Percoll gradient.

For trial experiments involving self-forming gradients, Percoll was diluted in 1% phenol-19% ethanol solution to give working solutions of 30% and 60% (v/v). Self-forming gradients were generated by centrifugation of 27 mL Percoll solution, prepared in Nalgene™ Oak Ridge High-Speed PPCO conical bottom centrifuge tubes, at 25,000 x *g* for 60 min at 4°C.

Cell suspensions used in Percoll density gradient centrifugation experiments were prepared as follows. For bacteria only or macrophage only samples, 3 mL suspensions containing 10^6 4/74 *rpsM*::*gfp*+ cells grown to ESP (as described in Section 2.6.1) or 10^7 RAW 264.7 macrophages were prepared in PBS and DPBS, respectively. 4/74 *rpsM*::*gfp*+ strain was used to aid visualisation of the bacterial cells along the Percoll gradient column. To simulate macrophage lysates, 10^7 macrophages were resuspended in 3 mL SDS-phenol-ethanol solution. Mixed *Salmonella*-macrophage lysates were prepared by mixing 10^6 4/74 *rpsM*::*gfp*+ cells and 10^7 macrophages in SDS-phenol-ethanol solution. The cell suspensions were loaded onto the pre-formed Percoll gradients, or mixed with the dilution Percoll solutions (for self-forming gradients), and centrifuged at 25,000 x *g*, at 4°C for 60 min.

To evaluate the effect of phenol-ethanol on Percoll gradient formation, Percoll (GE Healthcare Life Sciences) and Percoll PLUS were diluted in PBS or 1% phenol-19% ethanol solution to give working solutions of 30%, 40%, 50% and 60% (v/v). Self-forming gradients were generated as described above. Calibration of the Percoll gradients was achieved by the addition of density marker beads (DMBs) (Cospheric LLC, product code DMB-kit) prior to centrifugation of the Percoll solutions.

2.6.5 Comparison of RNA extraction methods

Four RNA extraction methods were compared for RNA yield and integrity: (1) TRIzol, (2) TRIzol Max Bacterial RNA Isolation Kit (Invitrogen), (3) RNAzol RT (Molecular Research Center, Inc.), and (4) an acidic phenol-chloroform extraction (A. Colgan, personal communication; Heptinstall, 1998; Jones *et al.*, 1994). *S.* Typhimurium 4/74 was grown to ESP (OD_{600} 2.0) as described in Section 2.6.1, and 1 mL of bacterial culture was used for each RNA extraction method. The remaining culture was serially diluted in PBS and plated onto LB agar plates for bacterial enumeration.

RNA extraction using TRIzol was performed as described in Section 2.6.3. RNA extractions using TRIzol Max Bacterial RNA Isolation Kit or RNAzol RT were carried out according to the manufacturer's instructions. For acidic phenol-chloroform extraction, the bacterial pellet was first resuspended in 0.5 mg/mL lysozyme in Tris-EDTA buffer at pH 8.0, followed by addition of 1% SDS and 100 µg/mL Proteinase K, and incubated at 40°C for 20 min. 3 M sodium acetate was then added to a final concentration of 0.3 M, followed by acidic phenol-chloroform solution (1:1) in equal volume to the entire reaction mixture. The mixture was incubated at 40°C for 5 min, followed by centrifugation at 14,000 rpm, 4°C for 15 min. Following centrifugation, the aqueous phase was transferred to a fresh 2 mL microcentrifuge tube, and an equal volume of 100% isopropanol was added. Samples were incubated on ice for 30 min to precipitate RNA, then centrifuged at 14,000 rpm, 4°C for 15 min. The supernatant was discarded and the pellet was washed with 500 µL 70% ethanol (in DEPC-treated water). The tube was centrifuged at 14,000 rpm, 4°C for 5 min. The ethanol was discarded, leaving behind the RNA pellet. RNA pellets were reconstituted in 20 µL of DEPC-treated water as described in Section 2.6.3.

2.6.6 Assessment of RNA yield and integrity

RNA concentrations (in ng/µL) were determined using the Qubit RNA Broad Range Assay (Life Technologies, product code Q10211) and Qubit 2.0 Fluorometer (Life Technologies), according to the manufacturer's instructions. RNA integrity was assessed using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer System. Intact *S.* Typhimurium RNA is indicated by the presence of three distinct bands corresponding to the *Salmonella* ribosomal RNA (rRNA) fragments (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979) in the Bioanalyzer gel image output.

2.7 Next-generation sequencing of *Salmonella* transposon mutant libraries

2.7.1 Construction of *S.* Enteritidis transposon mutant library

2.7.1.1 Transposome preparation

Libraries of transposon insertion mutants were constructed in *S*. Enteritidis strains P125109 and D7795 as previously described in Canals *et al.* (2019a), using the EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre, product code EZI982K). Briefly, a transposome mixture was prepared by mixing 1 µL glycerol, 1 µL TypeOne™ Restriction Inhibitor (Epicentre, product code TY0261H), 2 µL EZ-Tn5<KAN-2> transposon (at 0.1 pmol/µL) and 2 µL EZ-Tn5 Transposase and incubated at 22°C for 2 h. The transposome reaction was then divided into two and dialysed using a Millipore filter (0.025 µm pore size) with molecular grade H_2O for 30 min, increasing the total reaction volume to around 30–50 µL.

2.7.1.2 Preparation of bacterial cells for electroporation

Electro-competent cells were prepared as described in Section 2.4.4, except that 2 x 200 mL of cells were grown. When the desired OD_{600} was reached (OD_{600} 0.40 ± 0.05), bacterial cells were harvested and washed three times in half volume of ice-

cold 10% (v/v) glycerol. Cells were finally resuspended in 400 µL 10% glycerol, and then divided into 50 µL aliquots.

2.7.1.3 Mutagenesis of *S.* Enteritidis using EZ-Tn5 transposomes generated *in vitro*

Between 3 and 5 µL of the transposome reaction was added to each 50 µL of fresh electrocompetent bacteria. The mixture was incubated on ice for 5 min before transfer to a pre-chilled single use electroporation cuvette (2 mm gap). Electroporation was performed as described in Section 2.4.4. A total of eight electroporations with the transposome reaction and one control electroporation without transposome were performed.

Immediately after electroporation, 1 mL of pre-warmed SOC media was added to the electroporation cuvette and the cell suspension was transferred to a fresh 1.5 mL microcentrifuge tube. The electroporated cells and control cells were incubated at 37°C with shaking at 220 rpm. After 1 h, the contents from each microcentrifuge tube were plated on LB plates containing 50 µg/mL kanamycin (Km^{50}) (100 µL/plate, ~10 plates from each electroporation). After incubation overnight at 37°C, the number of colonies on each plate was estimated by counting a quarter of them, and from this the total number of colonies on all plates was estimated. To account for bacterial cell division during the 1 h recovery period, the number of cells before and after recovery was determined using serial dilutions from the control tube. The final number of transformant colonies was estimated to be 44,851 colonies for P125109 and 15,762 colonies for D7795, which can be inferred as the number of independent mutants in each transposon mutant library.

To create the frozen transposon mutant library stock, transformant colonies were scraped off plates in 1 mL LB per plate, pooled and grown overnight in a total volume

of 400 mL LB supplemented with Km^{50} in a 2 L flask at 37°C with shaking. Frozen stocks of the transposon library were prepared by mixing 900 µL of overnight culture and 600 µL of 50% (v/v) glycerol and multiple aliquots were stored at −80°C. Serial dilutions of the overnight culture were performed to calculate the number of cells in each stock aliquot of the transposon mutant library, which worked out to be 6.63 x 10^9 CFUs per P125109 transposon library stock aliquot and 9.24 x 10^9 CFUs per D7795 transposon library stock aliquot.

2.7.2 Passages of transposon library in LB, NonSPI2 and InSPI2

A 1.5 mL aliquot of P125109 or D7795 transposon library was grown in 25 mL LB + Km^{50} in a shaking water bath at 37°C, 220 rpm for 16 h. Cells harvested from 4 x 200 µL aliquots of the bacterial overnight culture were stored at −80°C until genomic DNA extraction to give the **input** sample (see Section 2.7.4). Another 1 mL of the bacterial culture was washed twice with PBS and resuspended in LB, NonSPI2 or InSPI2 media. A dilution 1:100 was inoculated into 25 mL of LB, NonSPI2 or InSPI2 media (without antibiotic), respectively, and cultures were incubated in a shaking water bath at 37°C, 220 rpm until ESP was reached (passage 1).

ESP was chosen for the analysis of the *S.* Enteritidis transposon libraries to allow most of the bacterial population to complete exponential growth, whilst minimising the chance of measuring effects of individual transposon insertions on competitive fitness. The choice of ESP also minimised the accumulation of secondary mutations in the bacterial culture (Navarro Llorens *et al.*, 2010). Prior experiments involving growth of P125109 and D7795 in LB, NonSPI2 and InSPI2 (B. M. Perez-Sepulveda, personal communication) have shown that the two strains have different growth rates in NonSPI2 and InSPI2. Growth curves were thus performed (as described in Section 2.3.2) to determine the time to reach the ESP growth phase (when growth of the

bacterial culture first reached a plateau) for the individual strains in the three media (**Table 2.5**).

Table 2.5 Early stationary phase (ESP) time-points for *S.* Enteritidis P125109 and D7795 growth in LB, NonSPI2 and InSPI2 media[a]

Strain	Post-inoculation time point (h) at which ESP was reached		
	LB	**NonSPI2**	**InSPI2**
P125109	7	10	10
D7795	6	24	24

[a] Growth conditions are described in Section 2.3.2

A total of two passages were performed in each media. For LB passages, 250 µL were transferred in each individual passage, following two washes with PBS. For NonSPI2 and InSPI2 passages, a culture volume with OD_{600} equivalent to the LB subculture inoculum were transferred into the subsequent passage e.g. if OD_{600} of LB passage 1 was 2 and OD_{600} of NonSPI2 passage 1 was 1, then 500 µL of NonSPI2 passage 1 would be used to inoculate passage 2. Cells from 4 x 200 µL aliquots of the second passage of LB (**output LB**), NonSPI2 (**output NonSPI2**) and InSPI2 (**output InSPI2**) were harvested and stored at −80°C until genomic DNA extraction (Section 2.7.4).

Two independent experiments for each media were performed. Where a transposon mutant library failed to grow adequately (which occurred most often in the InSPI2 acidic minimal media), the experiment was repeated with a new aliquot of the transposon library. This explains why additional input libraries are listed in **Table 2.6**, when a total of 16 libraries (2 inputs, 2 output LB, 2 output NonSPI2, 2 output InSPI2 per strain x 2 strains) would be expected from two independent runs of the experiment.

2.7.3 Infection of RAW 264.7 macrophages with transposon library

10^6 RAW 264.7 macrophage cells were seeded in each well of 6-well plates (Sarstedt) 24 h before infection. A 1.5 mL aliquot of P125109 or D7795 transposon library was grown in 25 mL LB + Km^{50} in a shaking water bath at 37°C, 220 rpm for 16 h, and genomic DNA was purified from two different biological replicates as **input** samples. OD_{600} of the overnight culture was measured and a bacterial inoculum with OD_{600} = 5.0 was prepared by pelleting and resuspending bacterial cells in appropriate volumes of DMEM supplemented with MEM NEAA and L-glutamine; this ensures that sufficient bacterial cells (~1.4 x 10^7 cells for P125109 and ~1.5 x 10^7 cells for D7795) representing the complexity of the transposon library were used in infection. Macrophages were infected at MOI of 5–10 with mouse serum-opsonised bacteria as described in Section 2.5.2. A total of 18 wells were used in each infection per strain, with 6 wells set aside to provide bacterial counts at 1.5 h and 12 h post-infection as described in Section 2.5.2, and calculating the fold-change replication of the intracellular bacteria (12 h versus 1.5 h).

At 12 h post-infection, macrophages were lysed with 1% Triton X-100. Macrophage lysates containing intracellular bacteria from 12 wells were pooled into one 15 mL centrifuge tube and centrifuged at 4,000 rpm for 5 min. The cell pellet was resuspended in 1 mL LB and transferred to a flask containing 24 mL LB supplemented with Km^{50} for 10 h growth at 37°C, 220 rpm (**output MAC**). To determine the effect of 10 h growth in LB on the transposon library, a fraction of the input library culture was sub-cultured in LB containing Km^{50} for 10 h (**output LB_10 h**). Cells were harvested from the output library cultures and stored at −80°C until genomic DNA extraction.

2.7.4 Illumina DNA library preparation and sequencing

Genomic DNA was purified from all input and output library cultures using the Zymo Research Quick-DNA Miniprep Plus Kit, following manufacturer's instructions. To

ensure sufficient genomic DNA for the preparation of Illumina DNA libraries, each DNA sample comprised DNA extracted from 4 x 200 µL aliquots of the respective bacterial cell samples. DNA concentrations (in ng/µL) were determined using the Qubit dsDNA High Sensitivity Assay and the NanoDrop 2000 spectrophotometer (Thermo Scientific).

2 µg of genomic DNA from each mutant pool was fragmented to an average size of 300–350 bp using the BioRuptor@Pico sonication system (15 s ON 90 s OFF, 9 cycles). Illumina DNA library preparation was performed using NEBNext® DNA Library Prep Master Mix Set for Illumina® (product code E6040L), following the manufacturer's instructions. Reaction products from each step of library preparation were purified using AMPure XP beads (Beckman Coulter).

To amplify the transposon-flanking region, transposon-specific forward primers (**Table 2.4**) were designed such that the first 10 bases of each Read 1 (R1) would be the transposon sequence. A unique 6-base barcode incorporated in the forward primer allowed for pooling of samples for sequencing in a single lane. 22 cycles of PCR (Canals *et al.*, 2019a; Langridge *et al.*, 2009) were performed with NEBNext Q5 Hot Start HiFi polymerase using the transposon-specific primers and the Illumina reverse primer PE PCR Primer 2.0 for each fragmented DNA sample, following the recommended denaturation, annealing and extension temperatures and durations for NEBNext Q5 Hot Start HiFi polymerase. The resulting DNA was quantified using Qubit dsDNA High Sensitivity Assay and visualised on an Agilent High Sensitivity DNA chip, following the manufacturer's instructions. Finally, the amplified library was purified with AMPure XP beads and eluted in 30 µL of molecular grade H_2O.

The list of Illumina DNA libraries generated in this study is given in **Table 2.6**. For sequencing, the Illumina DNA libraries from each strain were pooled in a ratio

corresponding to the difference in estimated library complexity (initially defined by the number of transformant colonies) between the two strains, to ensure that the higher complexity library will have more sequencing reads (see Section 2.7.1.3). The 12 DNA libraries from RAW 264.7 macrophage infection experiments were pooled in the ratio of 3:1 (P125109:D7795). The 42 amplified DNA fragment libraries from *in vitro* passages of transposon mutant libraries in LB, NonSPI2, InSPI2 (and chick infection experiments, details of which are not described in this thesis), were pooled in the ratio of 2:1 (P125109:D7795; the ratio was revised following sequencing of the 12 DNA libraries from macrophage infection experiments, where the actual library complexities were revealed to be ~200,000 unique insertions for P125109 and ~100,000 insertions for D7795).

QC assessment of the pooled DNA library and sequencing were performed by the Centre for Genomic Research (CGR), University of Liverpool. Each library pool was size-selected to 250–500 bp, then paired-end sequenced in one lane on HiSeq4000 at 2 x 150 bp (for macrophage infection samples) or two lanes on NovaSeq6000 (SP mode) at 2 x 150 bp (for *in vitro* and chick infection samples) respectively. 15% of the bacteriophage ϕX174 genome, provided by Illumina as a control, was added to each lane to overcome the low complexity of the bases that followed the barcode in R1.

Table 2.6 *S.* Enteritidis transposon library input and output samples generated and sequenced in this study

S/N	Sample	Primer used for final PCR	Designated category name in JBrowse (see Section 2.8.4.4)
Library pool sequenced on HiSeq4000			
1	P125109 input LB_1	PE PCR Tn-1	Old LB input
2	P125109 input LB_2	PE PCR Tn-3	
3	P125109 output LB 10 h_1	PE PCR Tn-2	Old LB output
4	P125109 output LB 10 h_2	PE PCR Tn-4	
5	P125109 output MAC_1	PE PCR Tn-5	Macrophage output
6	P125109 output MAC_2	PE PCR Tn-6	
7	D7795 input LB_1	PE PCR Tn-7	Old LB input
8	D7795 input LB_2	PE PCR Tn-8	
9	D7795 output LB 10 h_1	PE PCR Tn-9	Old LB output
10	D7795 output LB 10 h_2	PE PCR Tn-10	
11	D7795 output MAC_1	PE PCR Tn-11	Macrophage output
12	D7795 output MAC_2	PE PCR Tn-12	
Library pool sequenced on NovaSeq6000			
13	P125109 input	PE PCR Tn-1	New LB input
14	P125109 input_1	PE PCR Tn-23	
15	P125109 input_2	PE PCR Tn-26	
16	P125109 input_3	PE PCR Tn-29	
17	P125109 input_4	PE PCR Tn-32	
18	P125109 output LB_1	PE PCR Tn-24	New LB output
19	P125109 output LB_2	PE PCR Tn-27	
20	P125109 output NonSPI2_1	PE PCR Tn-25	NonSPI2 output
21	P125109 output NonSPI2_2	PE PCR Tn-28	
22	P125109 output InSPI2_3	PE PCR Tn-31	InSPI2 output
23	P125109 output InSPI2_4	PE PCR Tn-33	
24	D7795 input	PE PCR Tn-12	New LB input
25	D7795 input_1	PE PCR Tn-34	
26	D7795 input_2	PE PCR Tn-36	
27	D7795 input_3	PE PCR Tn-39	
28	D7795 input_4	PE PCR Tn-44	
29	D7795 output LB_1	PE PCR Tn-35	New LB output
30	D7795 output LB_2	PE PCR Tn-37	
31	D7795 output NonSPI2_3	PE PCR Tn-42	NonSPI2 output
32	D7795 output NonSPI2_4	PE PCR Tn-45	
33	D7795 output InSPI2_3	PE PCR Tn-43	InSPI2 output

Table 2.6 *(continued)* *S.* Enteritidis transposon library input and output samples generated and sequenced in this study

S/N	Sample	Primer used for final PCR	Designated category name in JBrowse (see Section 2.8.4.4)
***(continued)* Library pool sequenced on NovaSeq6000**			
34	D7795 output InSPI2_4	PE PCR Tn-46	
35	P125109 output LIV+SPL_1	PE PCR Tn-2	
36	P125109 output LIV+SPL_2	PE PCR Tn-3	
37	P125109 output LIV+SPL_3	PE PCR Tn-4	Spleen output
38	P125109 output LIV+SPL_4	PE PCR Tn-5	
39	P125109 output LIV+SPL_5	PE PCR Tn-6	
40	P125109 output CAE_1	PE PCR Tn-7	
41	P125109 output CAE_2	PE PCR Tn-8	
42	P125109 output CAE_3	PE PCR Tn-9	Caecum output
43	P125109 output CAE_4	PE PCR Tn-10	
44	P125109 output CAE_5	PE PCR Tn-11	
45	D7795 output LIV+SPL_1	PE PCR Tn-13	
46	D7795 output LIV+SPL_2	PE PCR Tn-14	
47	D7795 output LIV+SPL_3	PE PCR Tn-15	Spleen output
48	D7795 output LIV+SPL_4	PE PCR Tn-16	
49	D7795 output LIV+SPL_5	PE PCR Tn-17	
50	D7795 output CAE_1	PE PCR Tn-18	
51	D7795 output CAE_2	PE PCR Tn-19	
52	D7795 output CAE_3	PE PCR Tn-20	Caecum output
53	D7795 output CAE_4	PE PCR Tn-21	
54	D7795 output CAE_5	PE PCR Tn-22	

Note: Samples coloured in red were generated from chick infection experiments with the P125109 and D7795 transposon libraries and were sequenced together with samples from the *in vitro* passage experiments. The details of the chick infection experiments and data have been excluded from this thesis because they were not found to be biologically informative. Nevertheless, these samples are listed here for clarity, as the sequencing reads from input samples #13 and #24 were used in the combined analysis of all P125109 and D7795 input samples respectively.

2.8 Bioinformatic analyses

All bioinformatic analyses described in this study, with the exception of Cluster of Orthologous Genes (COG) categories assignment, were performed by Dr Alex Predeus. Sections 2.8.1, 2.8.3 and 2.8.4 were co-written by the Author and Dr Predeus.

2.8.1 *S.* Enteritidis genome sequences and annotations

The annotated complete genome assemblies of *S.* Enteritidis P125109 and D7795 used in this thesis are publicly available in the National Center for Biotechnology Information (NCBI) database. The accession numbers are SAMN16552335 (P125109) and SAMN16552336 (D7795) (Perez-Sepulveda *et al.*, 2021). Orthologues between the *S.* Enteritidis and other *Salmonella* strains presented in this thesis were identified using the pipeline described at https://github.com/apredeus/multi-bacpipe. Briefly, Roary (Page *et al.*, 2015) was used to find protein-coding orthologues. Following this, nucleotide BLAST (blastn) was used to find conserved non-coding RNAs, small proteins, and pseudogenes. Final validation of each annotation was done manually by comparing the annotations to the locus tags in the published P125109 annotation.

2.8.2 Assignment of Cluster of Orthologous Genes categories

eggNOG-mapper v2 (Huerta-Cepas *et al.*, 2017; Huerta-Cepas *et al.*, 2019) was used to assign Cluster of Orthologous Genes (COG) categories for the reference *S.* Enteritidis genomes, using the default parameters.

2.8.3 Analysis of *phoPQ* deletion mutants sequencing data

Snippy v3.2 pipeline (https://github.com/tseemann/snippy) was used to analyse the variants in the *phoPQ* deletion mutants in P125109 and D7795. Specifically, sequencing reads were mapped to the reference genome of respective strains using

bwa mem v0.7.15-r1140 (Li, 2013), after which the variants were called using freeBayes v1.1.0 (https://github.com/freebayes/freebayes). Aligned reads and variant calls were visualised using Integrative Genome Viewer (IGV) genome browser v2.4.11 (Robinson *et al.*, 2011). Large deletion of the *phoPQ* operon was not detected with the variant caller, but was confirmed by visual inspection of coverage in IGV. Other identified variants (two for P125109, five for D7795) were all homopolymer indels located in intergenic regions of the genome.

2.8.4 Bioinformatic processing of *S.* Enteritidis transposon insertion data

Bioinformatic processing and analysis of *S.* Enteritidis transposon insertion data followed the general strategy described in Langridge *et al.* (2009) and later formalised as Bio-Tradis analysis (Barquist *et al.*, 2016). Several alterations specific to the data in hand similar to approaches used in Canals *et al.* (2019a) were implemented by Dr Predeus. The scripts and full description of the used pipeline are available at https://github.com/apredeus/TRADIS. Briefly, we performed essentiality analysis using unique transposon insertion counts, but we have adjusted the distribution fitting strategy to accommodate for data with fewer unique insertions. Comparative fitness analysis was done using deduplicated read counts from the same alignment. We have also changed the pipeline to streamline the genomic track visualisation in JBrowse.

2.8.4.1 Sequence analyses of transposon library

Raw sequencing data was demultiplexed using cutadapt v2.6 (Martin, 2011). First, a barcode sequence fasta file that included one sequence per sample was compiled, after which cutadapt was run with options “cutadapt -O 34 -g file:barcodes.fa --discard-untrimmed”. This generated a set of two paired-end fastq files for each sample. The reads were then aligned to the reference genome using bwa v0.7.17-r1188 (using bwa mem algorithm). Aligned BAM files were sorted and indexed using samtools v1.9 (Li *et al.*, 2009).

For further processing, two GFF annotation files were generated for each bacterial strain used in the experiments. One file was used for deduplicated read counting and was obtained from a general annotation file by changing the type of each feature that had a locus tag (ID) into “gene”. The second GFF file was generated the same way, with additional change to the annotated feature size: the last 10% of each annotated gene was removed. This annotation file was used in essentiality analysis, since previous reports (Barquist *et al.*, 2016; Goodall *et al.*, 2018) showed that insertions or deletions in the last 10% of the gene are much less likely to cause a complete loss of function.

Following these steps, a series of additionally processed alignment (BAM) files was created. First, picard MarkDuplicates v2.21.2 was used to remove PCR and optical duplicates from the aligned reads. The resulting files were filtered using *cigar_filter.pl* to select only the reads that align exactly at the start of R1 without softclipping. The resulting filtered BAM files were converted into 1 nucleotide single-end BAM files using *make_1nt.pl* script to avoid counting reads that spanned into the nearby genes.These alignment files were used for quantification of deduplicated reads and used in DESeq2 analysis. In parallel, the filtered BAM files were also converted into 1 nt unique insertion BAM files using *make_1nt_uniq.pl* script; these files were used for essentiality analysis.

The resulting 1 nt BAM files were quantified using featureCounts v1.6.4 (Liao *et al.*, 2014) with “-M -O --fraction -t gene -g ID -s 0” options for DESeq2 analysis, and “-M -O -t gene -g ID -s 0“ options for essentiality analysis. This was done to account for multimapping reads: if only uniquely mapping reads are considered, transposable and other repetitive elements look falsely essential. Indeed, if multimapping reads are discarded, genes that have many copies (such as transposons) appear to have zero

transposon insertion rate, which in turn leads us to the false conclusion of their essentiality.

2.8.4.2 DESeq2-based fitness analysis

Sometimes a transposon insertion does not cause an phenotype in one environment, but can cause a reduction in fitness of the strain under certain conditions — e.g. inside macrophages. In order to identify the genes that cause such differences, we profiled two *S.* Enteritidis strains under multiple growth conditions. Analysis of differential fitness for each strain was performed in R v4.0.2 using DEseq2 v1.28.1 (Love *et al.*, 2014) and a simple study design (~ Condition) with default settings. Deduplicated raw read counts were used as expression values. Each condition was represented by at least two biological replicates. Results are shown in log2 fold-change. A cut-off of 2-fold-change and *P*-value < 0.05 was applied.

2.8.4.3 Essentiality analysis

Essentiality analysis was done using the unique insertion counts. For consistency with our DESeq2-based fitness analysis, we have re-implemented the essentiality analysis functions used in Bio-Tradis (Barquist *et al.*, 2016; specifically R functions *make_ess_table* and *calculate_essentiality*). Briefly, the unique insertion counts were converted into insertion index (that is, insertion sites divided by gene length) (Langridge *et al.*, 2009), which followed the expected bimodal distribution. The distribution histogram was used to fit two functions: exponential function for very low insertion indices corresponding to the required genes, and gamma function for high insertion indices of the dispensable genes. Using the obtained fits, the genes were classified as follows: if log-likelihood of the exponent to gamma distribution was 2 or higher, the gene was deemed “required”; if the ratio was below −2, it was deemed “not required”; genes with intermediate insertion indices were reported as

"ambiguous". Due to the lower insertion densities in our libraries essentiality calls were not assigned for features shorter than 200 nt.

It should be noted that the scripts available at https://github.com/apredeus/TRADIS used the terms "essential" to describe genes with very low insertion indices and "non-essential" for genes that are dispensable. However, in this thesis, the terms "required" and "not required" were adopted instead, for reasons explained in Section 1.6.

2.8.4.4 Visualisation

The alignments, unique insertions, and essentiality calls were visualised in JBrowse 1.16.5 (Buels *et al.*, 2016). To visualise the insertions, GFF files of unique insertions and of genes with essentiality annotations were generated for each sample. The data tracks are available for online visualisation at **hintonlab.com/jbrowse/index.html?data=tradis_P125109/data** and **hintonlab.com/jbrowse/index.html?data=tradis_D7795/data** respectively.

2.8.4.5 Inter-strain essentiality comparison

Data from the *S.* Typhimurium D23580 transposon library (Canals *et al.*, 2019a) was used as a comparator to identify common *Salmonella* genes that were required for growth under laboratory conditions or during macrophage infection, and identify differences that may reflect unique requirements for each serovar or the pathogenic niche these bacteria inhabit. Required gene comparison between *S.* Enteritidis strains P125109 and D7795, and D23580 was complicated by substantial differences in the average number of unique insertions present in the transposon library for each strain. To identify robust differences, we used both deduplicated read counts and essentiality calls. Deduplicated read counts for LB input, LB output, and macrophage output for the three strains were log2-transformed and quantile-normalised. These values were then used to calculate log2 fold-change that was evaluated and found to follow

approximately normal distribution (data not shown). Thus, we have selected the genes that were significantly different in all three conditions in different strains according to t-test (*P*-value ≤ 0.05) and also had differing essentiality calls (see Section 2.8.4.3). This allowed us to identify 63 genes that satisfied both conditions. Resulting genes were visualised using Phantasus gene expression analysis tool (http://genome.ifmo.ru/phantasus-dev/). K-means clustering of rows allowed us to identify five groups of genes according to the strain they were essential in.

2.9 Statistical analyses

Statistical analyses were done with GraphPad Prism 7 (version 7.04). Ordinary one-way ANOVA and the Bonferroni's multiple comparison test were used to determine differences between data sets. A *P*-value of less than 0.05 was considered to be statistically significant.

Chapter 3 :

Optimising isolation of *Salmonella* RNA from infected macrophages

3.1 Introduction

An important, and perhaps the most challenging aspect of research into bacterial pathogenesis is to understand the metabolic interactions between a pathogen and its host during infection (Smith, 2000). The study of gene expression and determining how these transcripts are regulated during infection provide a window into understanding host-pathogen interactions. Various studies have shown that genes highly upregulated during infection play important roles in adaptation, survival, and proliferation within mammalian cells, and are more likely to play a direct role in pathogenesis (Hammarlöf *et al.*, 2013; Hammarlöf *et al.*, 2018; Srikumar *et al.*, 2015). Transcriptomic approaches involving DNA microarrays or RNA sequencing (RNA-seq) allow the simultaneous monitoring of most (if not) all genes in an organism, and have been used to define the gene expression profiles of various intracellular pathogens such as *Listeria monocytogenes* (Chatterjee *et al.*, 2006), *Mycobacterium tuberculosis* (Schnappinger *et al.*, 2003), *S.* Typhimurium (Canals *et al.*, 2019b; Eriksson *et al.*, 2003; Hautefort *et al.*, 2008; Srikumar *et al.*, 2015) and *Shigella flexneri* (Lucchini *et al.*, 2005) during infection.

Regardless of the technology used, a robust transcriptomic analysis depends on the yield, integrity and purity of the RNA used in the experiment. Before the advent of dual RNA-seq, which allows the simultaneous RNA-seq analysis of a pathogen and its infected host (Westermann *et al.*, 2012), transcriptional analysis of intracellular pathogens such as *Salmonella* typically involved the isolation of bacterial cells from the host. The bacterial transcriptome (comprised of messenger [mRNAs] and small RNAs [sRNAs]), forms a small percentage of total bacterial RNA (La *et al.*, 2008; Westermann *et al.*, 2012); published RNA-seq transcriptomic analysis of *S.* Typhimurium grown *in vitro* suggests the *Salmonella* mRNA and sRNA makes up 36% of total *Salmonella* sequence reads (Kröger *et al.*, 2012). In the context of an infected cell, host components (DNA, RNA, proteins) are present in huge excess

relative to bacterial RNA (Westermann *et al.* [2012] estimated that eukaryotic RNA alone present in samples derived from infected cells is approximately 20 times the amount of bacterial RNA), necessitating an efficient method to separate bacterial RNA from these host components. Bacterial RNA is extremely labile, with 80% of *E. coli* mRNAs having half-lives between 3 and 8 min (Bernstein *et al.*, 2002). Consequently, RNA must be stabilised and protected from degradation by RNases during the RNA isolation process (Hinton *et al.*, 2004). Furthermore, bacteria are highly adaptable and sensitive to environmental changes (Kröger *et al.*, 2013; La *et al.*, 2008; Rolfe *et al.*, 2012), and any delays in the harvesting of bacterial RNA could result in changes in the transcriptome.

In 2003, the Hinton Laboratory developed and published a differential lysis approach involving an 'RNA stabilisation solution' that contains 0.1% sodium dodecyl sulphate (SDS), 1% acidic phenol and 19% ethanol to study the intracellular gene expression profile of *S.* Typhimurium during infection of murine macrophages (Eriksson *et al.*, 2003). SDS, used at a low concentration, lyses the macrophage cells without affecting the intracellular bacteria, and acidic phenol-ethanol serves to halt transcription and inhibit RNase activity (McDowall *et al.*, 1994). This differential lysis approach of isolating intracellular *Salmonella* RNA has since been modified (summarised in **Table 3.1**), and the protocol described in Srikumar *et al.* (2015) (**Figure 3.1**) is the basis for all experiments described in this chapter. Although the differential lysis approach has been used successfully to define the *Salmonella* transcriptome during infection of epithelial cells (Hautefort *et al.*, 2008) or macrophage cells (Canals *et al.*, 2019b; Srikumar *et al.*, 2015), researchers involved in these studies have noted (in personal communication) that the protocol is technically challenging and does not always yield *Salmonella* RNA, or generates insufficient amounts of RNA for reliable transcriptomic analyses. Eukaryotic RNA contamination of the *Salmonella* RNA is also a common problem.

The assessment of bacterial RNA quality is commonly achieved by the visualisation of rRNA by capillary electrophoresis on an Agilent 2100 Bioanalyzer System (Section 2.6.6). Most Gram-negative bacterial species including *E. coli* possess three forms of rRNA, namely 23S, 16S and 5S (Smith *et al.*, 1988). To interpret electropherograms of *Salmonella* rRNA, it is important to know that the 23S rRNA of *S.* Typhimurium is cleaved into two smaller molecules (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979). *S.* Enteritidis, on the other hand, does not display the same 23S rRNA cleavage behaviour as *S.* Typhimurium.

Initially, we planned to perform RNA-seq-based transcriptomic analyses of Global Epidemic and African *S*. Enteritidis during macrophage infection, with the aim of identifying genes that exhibit differential expression patterns unique to African *S.* Enteritidis. This chapter describes a body of work designed to optimise our intra-macrophage *Salmonella* RNA extraction protocol, in the hopes of increasing the bacterial RNA yield and improving the technical repeatability of the protocol.

The experimental work in this chapter was cut short by the COVID-19 pandemic of 2020/2021, and the associated closure of the University of Liverpool research laboratories. I had planned to complete the experiments for optimising the extraction of *Salmonella* RNA from infected macrophages by quantifying RNA using quantitative reverse transcription PCR (RT-qPCR) in a large parallel assay as one of the last experiments of my PhD project. Unfortunately, it was not possible to optimise the RT-qPCR conditions or to perform the final quantification of the RNA which would have allowed a definitive comparison between the different methods (presented in this chapter) to be done.

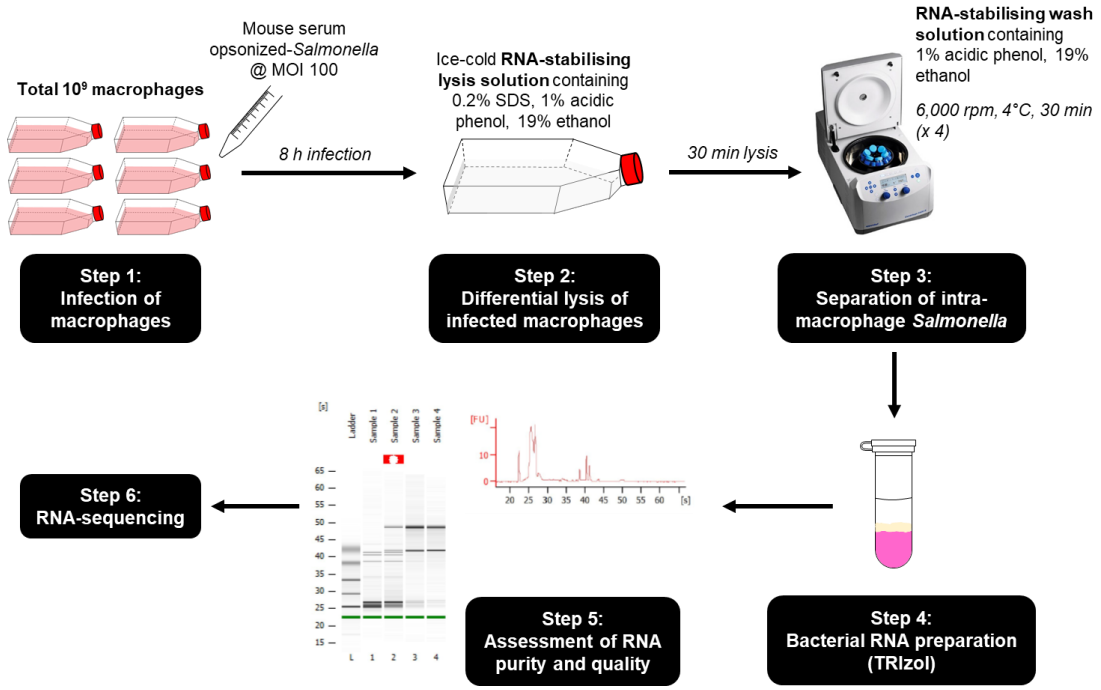

Figure 3.1 Schematic representation of the Hinton Laboratory intracellular *Salmonella* RNA extraction protocol

The workflow shown was used in the RNA-seq-based transcriptomic studies of *S.* Typhimurium during murine macrophage infection (Canals *et al.*, 2019b; Srikumar *et al.*, 2015). Key details of the protocol are highlighted in **Table 3.1**.

Table 3.1 Modifications of the Hinton Laboratory differential lysis approach for isolating *Salmonella* RNA from infected mammalian cells (2003–2019)

Publication	Key differences in protocol		
	Step 1: Infection	**Step 3: Separation of intra-macrophage *Salmonella***	**Step 4: RNA extraction**
Eriksson *et al.* (2003)	• 10^8 J774-A.1 murine macrophage cells • 6-well cell culture plates, 20 plates in total • 5-min centrifugation at 1,000 x *g* following bacteria inoculation	• Lysis solution: 0.1% SDS, 1% acidic phenol, 19% ethanol • Centrifugation of *Salmonella*-containing macrophage lysates in microcentrifuge tubes at 14,000 rpm	• Promega SV Total RNA purification kit • Bacteria RNA further purified by phenol-chloroform extraction
Hautefort *et al.* (2008)	• 10^8 HeLa cells • 75 cm^2 tissue culture flasks, 10 flasks in total • 5-min centrifugation at 1,500 rpm following bacteria inoculation	• Lysis solution: 0.1% SDS, 1% acidic phenol, 19% ethanol	• Promega SV Total RNA purification kit • Bacteria RNA further purified by phenol-chloroform extraction
Srikumar *et al.* (2015)	• 10^9 RAW 264.7 murine macrophage cells • 175 cm^2 tissue culture flasks, 6 flasks in total • No centrifugation step following bacteria inoculation	• Lysis solution: 0.2% SDS, 1% acidic phenol, 19% ethanol • Centrifugation of *Salmonella*-containing macrophage lysates in 50 mL centrifuge tubes at 6,000 rpm	• TRIzol-based RNA extraction
Canals *et al.* (2019b)	Same protocol as Srikumar *et al.* (2015)		

3.2 Results

Experiences with the intra-macrophage *Salmonella* RNA isolation protocol shared by past and present Hinton Laboratory colleagues strongly indicated that the separation of intra-macrophage *Salmonella* from lysed macrophages (**Figure 3.1**, Step 3) is the major bottleneck in the successful isolation of bacterial RNA. Other limiting factors, such as the intra-macrophage *Salmonella* bacterial numbers at 8 h post-infection (Step 1) and the choice of RNA purification method/kit (Step 4), were also suggested. To improve the intra-macrophage *Salmonella* RNA extraction protocol, I made different modifications to the aforementioned three steps in the protocol. Experiments were conducted using *S.* Typhimurium strain 4/74 and RAW 264.7 macrophages to determine the impact of the modifications. The factors taken into consideration when deciding on a modification included compatibility with critical components of our protocol (e.g. the use of phenol-ethanol), the ease of operation, and the amount of time the modification may add to or subtract from the existing intra-macrophage *Salmonella* RNA isolation protocol.

3.2.1 Reintroduction of brief centrifugation of cell cultures following bacterial inoculation

Frequently, only a minor fraction of host cells in a sample will be infected. For example, Westermann *et al.* (2016) observed that approximately 2–5% of HeLa cells were infected with *Salmonella* in their study. Increasing the proportion of infected cells could translate to higher total intracellular bacteria numbers, and therefore a higher bacterial RNA yield.

Using a high MOI (such as 100:1 in our infection protocol) or a brief centrifugation of the cell culture plates (or flasks) following bacterial inoculation are common techniques to maximise bacterial contact, thereby increasing chances of uptake and therefore infection (Achouri *et al.*, 2015; Gog *et al.*, 2012). As described in **Table 3.1**,

the Srikumar *et al.* (2015) protocol used 175 cm^2 tissue culture flasks (T175) for macrophage infection, compared to 6-well plates in the original Eriksson *et al.* (2003) protocol. The use of the larger T175 flasks increased the total number of mammalian cells that can be used for each infection (10^8 to 10^9) while reducing the number of cell culture vessels for easier handling (twenty 6-well plates to six T175 flasks). However, the brief centrifugation step following bacteria inoculation had to be omitted, as the T175 flasks were too big to fit into standard size centrifuge adaptors for cell culture flasks (Eppendorf, product code 5825719000), which were designed for the smaller T75 (and T25) tissue culture flasks. I investigated if this omission of the centrifugation step could affect amount of bacterial uptake by macrophages and therefore total intra-macrophage *Salmonella* numbers at 8 h post-infection.

To determine the impact of the centrifugation step on bacterial uptake by macrophages and levels of intra-macrophage replication, intra-macrophage *Salmonella* replication assays (described in Section 2.5.2) were performed using T75 and T175 flasks. Replacing the T175 flasks with T75 flasks and re-introducing a 5-min centrifugation step resulted in higher bacterial uptake at 1.5 h post-infection (**Figure 3.2A**) and greater intra-macrophage *Salmonella* numbers at 8 h post-infection, although there was no statistical significance between the bacterial yields (**Figure 3.2B**). Additionally, the smaller flasks were easier to handle than the larger flasks during the process of scraping off the lysed macrophages (Step 2). Taken together, the use of T75 flasks and a brief centrifugation step should help to increase the total yield of intra-macrophage *Salmonella* cells; these changes were made to the Srikumar *et al.* (2015) protocol. For the rest of this chapter, the 'current' protocol refers to the use of eight T75 flasks (unless otherwise stated) of macrophages in a single infection and a 5-min centrifugation at 1,000 rpm after the addition of bacteria (as described in Section 2.6.2).

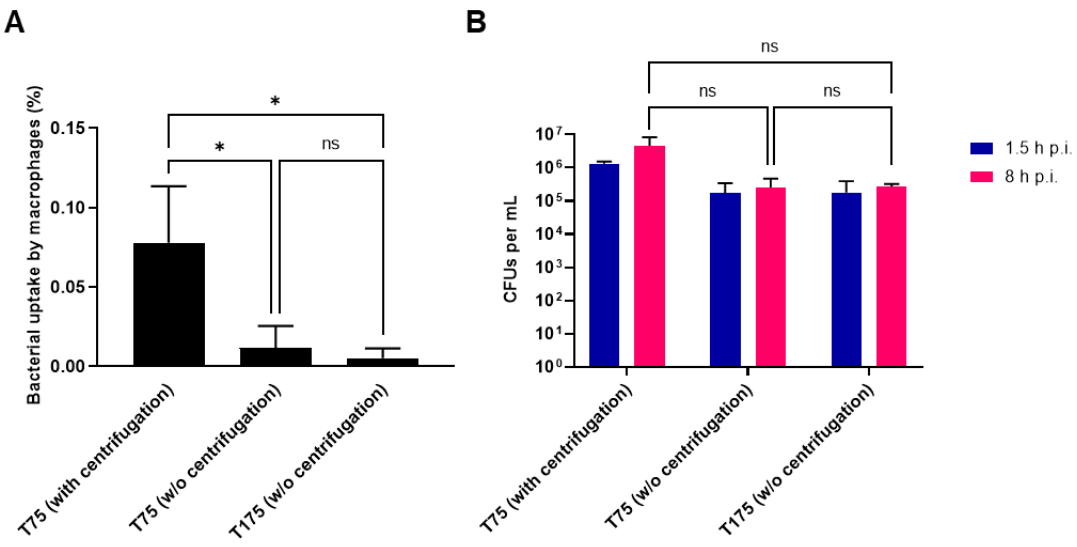

Figure 3.2 Effect of centrifugation step in infection protocol on *Salmonella* uptake and replication in RAW 264.7 macrophages

RAW 264.7 macrophages were seeded in T75 or T175 flasks and infected with *S.* Typhimurium 4/74 as described in Section 2.5.2. **A.** Bacterial uptake was measured by viable counts of intracellular bacteria at 1.5 h post-infection (p.i.), and is expressed as a percentage of the inoculum used in the infection. **B.** Intra-macrophage *Salmonella* numbers at 8 h p.i. represented as colony forming units (CFUs) per mL, determined by the Miles and Msra method described in Section 2.3.1. Data represents the mean of three independent experiments with two replicates each, and error bars show standard deviation. Abbreviations: T75, 75 cm^2 tissue culture flasks; T175, 175 cm^2 tissue culture flasks; w/o, without; ns, non-significant, *P*-value ≥ 0.05; *, *P*-value < 0.05.

3.2.2 Physical capture of bacteria by filtration

As already mentioned, a major limitation of our differential lysis approach for the isolation of intra-macrophage *Salmonella* RNA is the inefficient separation of the released bacteria from host debris by centrifugation (**Figure 3.1**, Step 3). The first alternative method for intra-macrophage *Salmonella* separation chosen for testing and comparison against our current protocol was a filtration-based method described by Sigal *et al.* (2016), owing to its apparent ease of use. In this filtration-based method, *Listeria monocytogenes* was isolated from infected macrophages by capturing intracellular bacteria on a 0.45 µm filter through vacuum-driven filtration of the macrophage lysates.

Eight T75 flasks of RAW 264.7 macrophages were infected as described in Section 2.6.2, and four flasks were processed using each method. RNA extracted from each method was compared by capillary electrophoresis. Results showed that the differential centrifugation method generated more intra-macrophage *Salmonella* RNA than the filtration method (**Figure 3.3A**). The RNA sample prepared from bacterial cells purified by filtration contained mostly eukaryotic RNA, whereas the RNA sample prepared from bacterial cells purified by centrifugation contained a mixture of intact *Salmonella* and eukaryotic RNA (evidenced by the sharp, distinct bands and absence of smears indicative of RNA degradation).

A possible reason for the presence of predominantly eukaryotic RNA in the sample prepared by the filtration method could be the clogging of the 0.45 µm filter by the viscosity by the macrophage lysate: it was extremely difficult to pass about 40 mL of macrophage lysate through a single filter. The experiment was thus repeated using multiple filters, passing a smaller volume of the macrophage lysate through each filter. The bacteria-containing solutions generated from the washing of each filter were then pooled for RNA extraction. However, insufficient bacteria were recovered by this

method of using separate filters, and an extremely low level of RNA was extracted (**Figure 3.3B**).

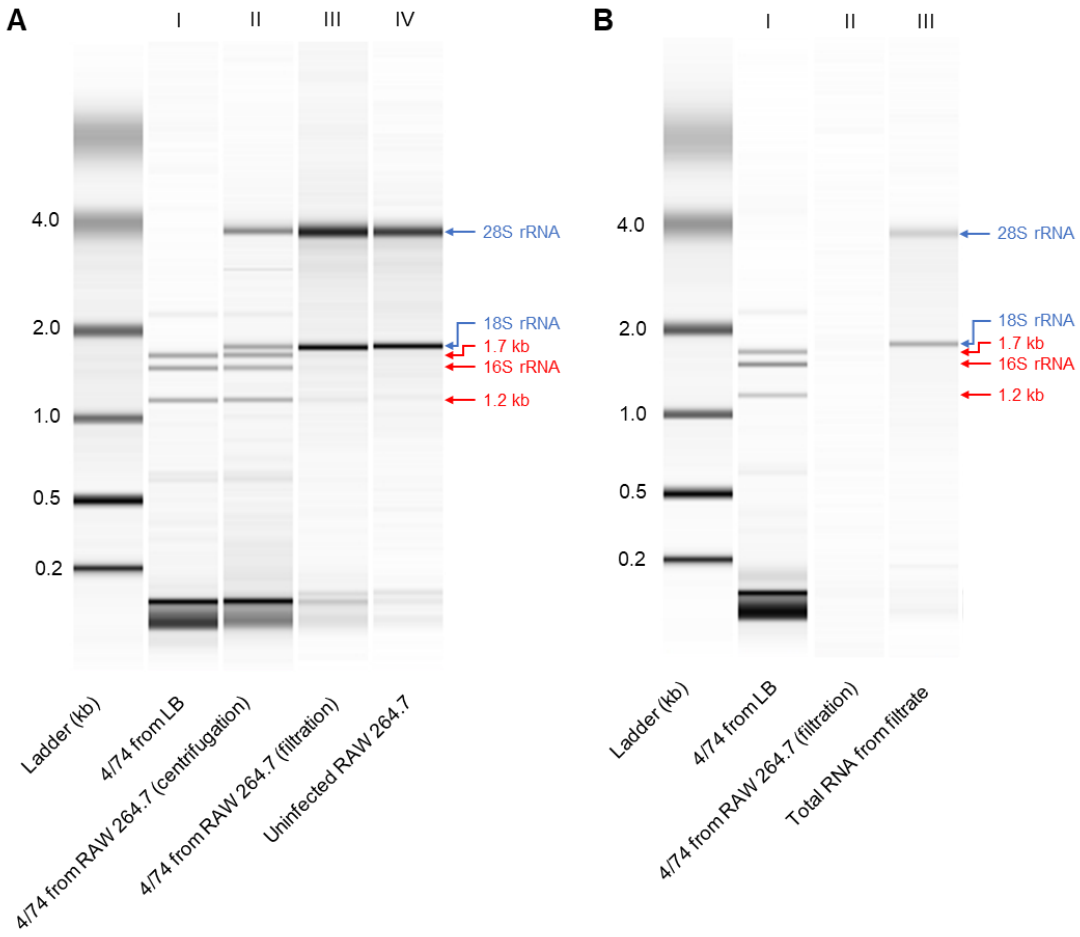

Figure 3.3 Filtration of *Salmonella*-containing macrophage lysate using 0.45 µm filter did not improve the separation of intra-macrophage bacteria from macrophage cell debris

A. Total RNA was extracted from (I) *S.* Typhimurium 4/74 grown in LB *in vitro*, (II) intra-macrophage 4/74 separated by differential centrifugation method, (III) intra-macrophage 4/74 separated by filtration method, and (IV) uninfected RAW 264.7 macrophages, as described in Section 2.6. Eight T75 flasks of macrophages were infected as described in Section 2.6.2, and four flasks of infected macrophages were processed using each method. Lanes I and IV represent the expected output for intact and pure RNA extracted from *Salmonella* and RAW 264.7 cells respectively. **B.** The filtration method was repeated with eight T75 flasks of infected macrophages, with four separate filters and each filter receiving a smaller volume of macrophage lysate. The samples were then pooled for RNA extraction, with (II) RNA from intra-macrophage *Salmonella*, and (III) RNA from the filtrate. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.2.3 Percoll gradient centrifugation

Our current intra-macrophage *Salmonella* RNA isolation protocol uses differentiation centrifugation, that is, the separation of *Salmonella* from lysed macrophages/cell debris primarily on the differences in their particle size (Rickwood, 2001). In contrast, density gradient centrifugation uses a supporting column of liquid with density, concentration, or viscosity that increases towards the bottom of the tube, to separate a mixture of components based on their densities (Graham, 2001).

One commercially available density gradient medium is Percoll (GE Healthcare Life Sciences), which is a colloidal suspension of polyvinylpyrrolidone-coated silica particles of 15–30 nm in diameter with a density of 1.130 g/L. The particles of varying sizes sediment at different rates when centrifuged, resulting in the formation of a gradient. Percoll has been used to purify bacterial sub-populations from *E. coli* cultures (Makinoshima *et al.*, 2002), or obligate intracellular bacteria from infected cell cultures (Lis *et al.*, 2014; Tamura *et al.*, 1982; Yuksel *et al.*, 2006). Consequently, I investigated whether Percoll could be used for the separation of *Salmonella* from lysed macrophages.

Percoll gradients can be used either as pre-formed gradients, or self-generating gradients, which involved mixing the sample to be separated directly with Percoll solution and the Percoll gradient is formed *in situ* when the mixture is centrifuged together. Using a Green Fluorescent Protein (GFP)-expressing strain of 4/74 (4/74 *rpsM::gfp*+), I first performed trial experiments to test both types of gradients for the purposes of *Salmonella* separation **(Figure 3.4)**. Results obtained suggested that *Salmonella*, intact macrophages, and macrophage lysates had similar buoyant densities, as indicated by their similar positions along the density gradient.

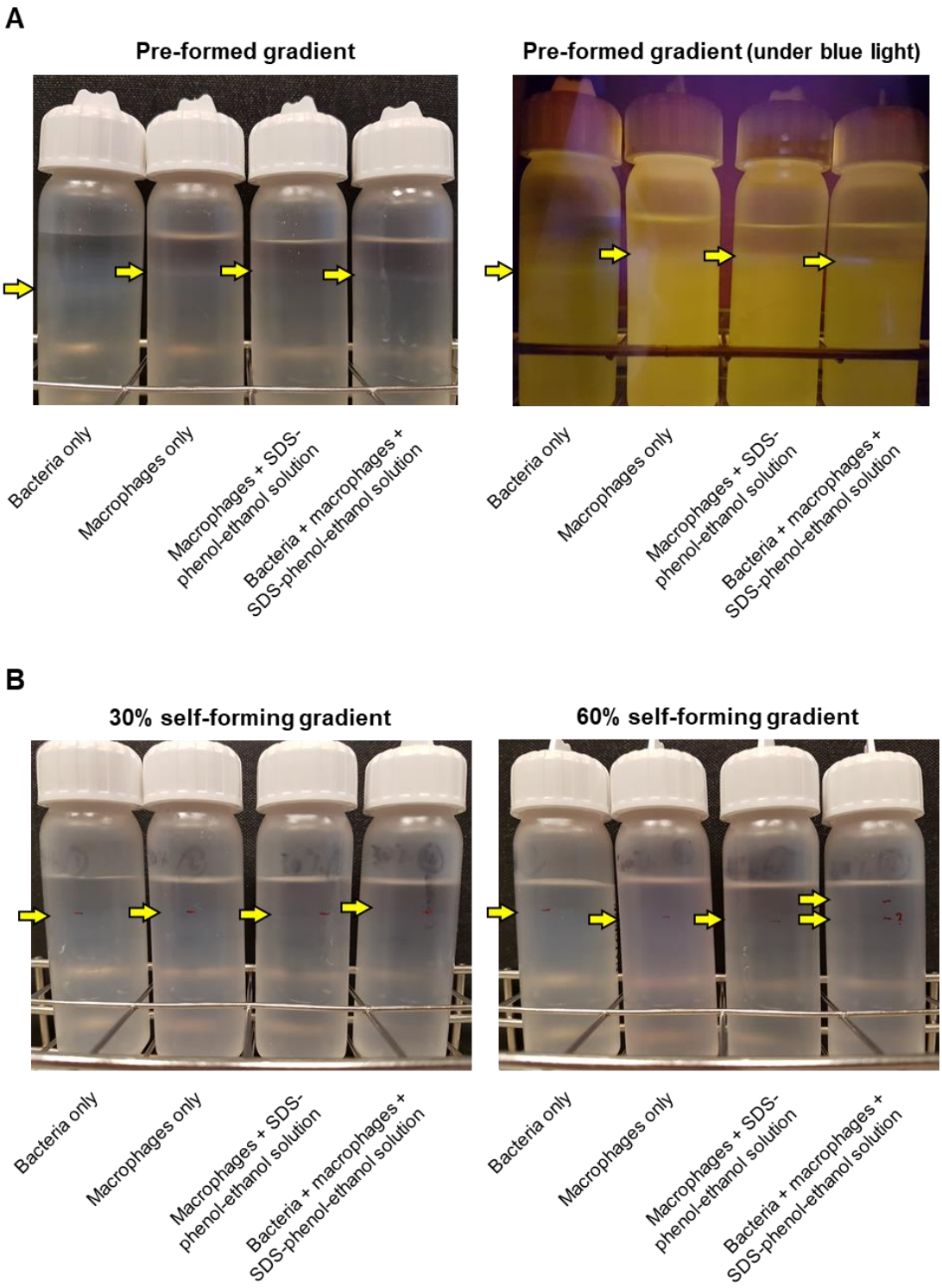

Figure 3.4 Trial experiments of Percoll gradient centrifugation of *Salmonella* cells, macrophages, and *Salmonella*-macrophage lysate mixed samples

Samples used in separation were prepared as described in Section 2.6.4.6. **A.** Pre-formed Percoll gradient as described in Section 2.6.4.6. The yellow arrows indicate the probable band position of the respective components. Right panel shows the tubes illuminated under blue light, but the fluorescence of the bacterial band was not obvious compared to background. **B.** Percoll solution was diluted to 30% (v/v) and 60% (v/v) solutions and mixed with the respective samples directly and centrifuged. In this experiment, the band(s) within each centrifuge tube could not be clearly identified.

Percoll gradients are commonly prepared in sucrose or saline solutions; however, using Percoll to separate *Salmonella* cells to be used for RNA preparation necessitated the use of an RNA-stabilising agent such as phenol-ethanol. To determine if phenol-ethanol affected the formation of Percoll gradients, self-generating gradients were made using a range of Percoll solutions prepared in PBS and phenol-ethanol, and the gradient shapes (i.e. variation of concentration along the tube) were visualised with DMBs (**Figure 3.5**). Results indicated that the use of phenol-ethanol to prepare Percoll gradients greatly altered the Percoll gradient characteristics: compared to conventional Percoll-PBS gradients which showed a gradual change in the density along the length of the gradient column, the density distribution in Percoll-phenol-ethanol gradients was mostly concentrated towards the bottom of the centrifuge tube (**Figure 3.5B**).

Subsequent centrifugation experiments found that the buoyant density of *Salmonella* cells in Percoll-phenol-ethanol solution was in the range of 1.08 (dark blue DMB) – 1.10 g/mL (red DMB) (data not shown). Considering that this density range would be located towards the bottom of Percoll density gradient (**Figure 3.5B**), and that *Salmonella* cells, intact macrophage cells and macrophage cellular debris all appeared to have similar buoyant densities (**Figure 3.4**), it is highly unlikely that *Salmonella* cells can be purified away from the macrophage cellular debris (or any intact macrophages that remained after SDS lysis).

The observed differences between Percoll-phenol-ethanol and Percoll-PBS gradients were initially thought to be a result of the effect of phenol on the Percoll medium, as direct addition of phenol caused the Percoll particles to aggregate due to lowering of pH (**Figure 3.5C**) (this aggregation effect was circumvented by using a solution of 1% phenol-19% ethanol rather than phenol alone). However, similar Percoll gradient

profiles were obtained when Percoll PLUS, the more chemically stable version of the product, was used (**Figure 3.5D**).

I deduced that the difference in the solution densities of the 1% phenol-19% ethanol (0.957 ± 0.030 g/mL) and PBS (1.003 ± 0.003 g/mL) were responsible for the issue because of the disparity between the initial Percoll densities (1.043 g/mL vs. 1.066 g/mL). Following centrifugation, a gradient with a density of 1.043 g/mL would have a completely different profile to a gradient with a density of 1.066 gm/L (GE Healthcare Life Sciences and Sciences, 2014).

Taken together, the findings in this section led me to conclude that Percoll gradient centrifugation was not a suitable replacement for differential centrifugation.

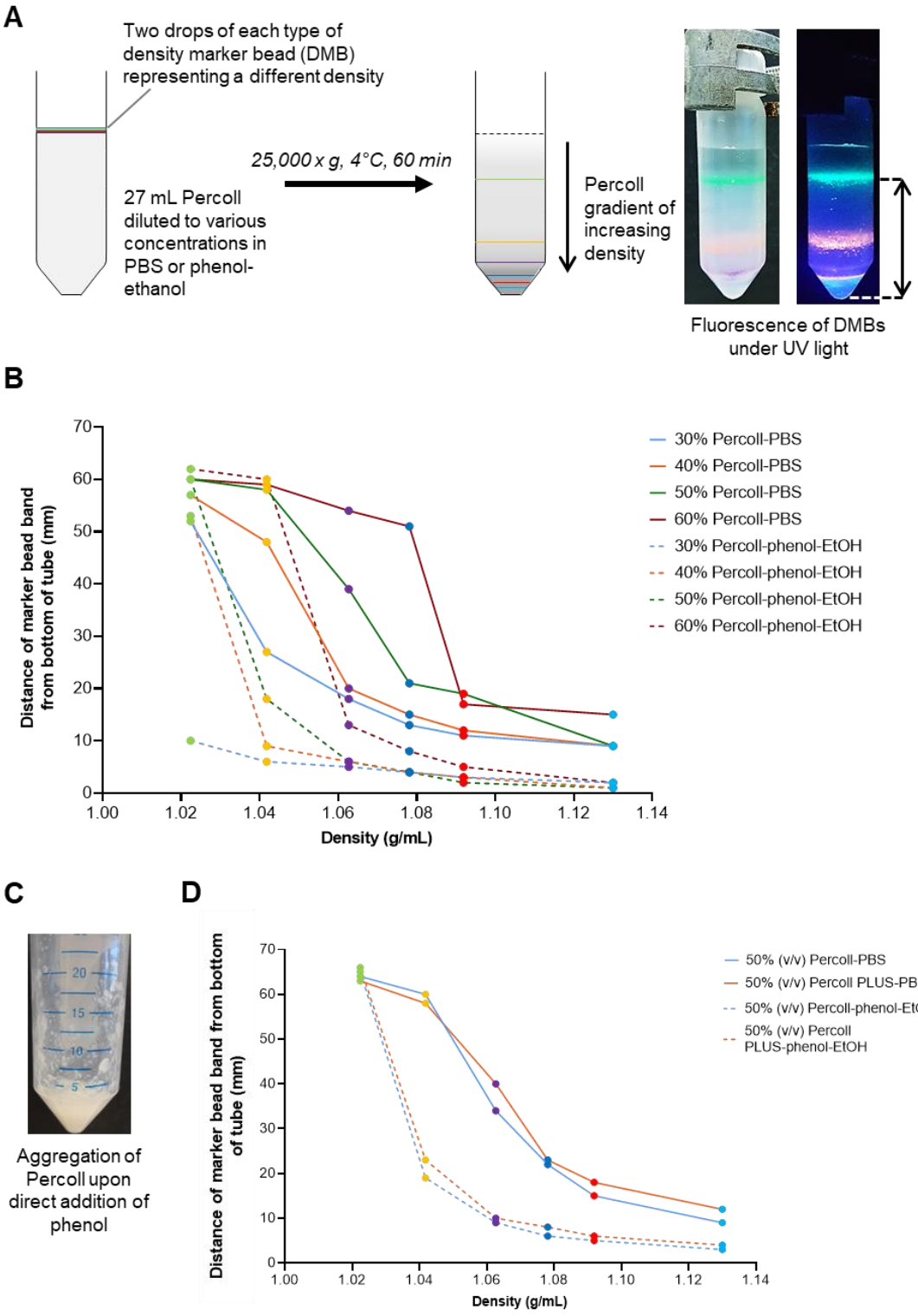

Figure 3.5 Phenol-ethanol alters the properties of Percoll gradients

A. Percoll solutions were diluted to concentrations ranging from 30% (v/v) to 60% (v/v) in PBS or 1% acidic phenol-19% ethanol (EtOH) solution. Self-forming gradients were generated by centrifugation of the respective Percoll solutions at 25,000 x *g*, 4°C for 60 min. Density marker beads (DMBs) (Cospheric LLC) of known mass density were used to calibrate the gradient, and form a band at a position corresponding to the specified density along the gradient column. Fluorescence of the marker beads under UV light permitted visualisation of the bands.

B. Percoll gradient profiles obtained with varying concentrations of Percoll prepared in PBS or 1% phenol-19% EtOH, visualised by plotting the distance between a DMB band and the bottom of the centrifuge tube (*y* axis) against density (*x* axis). Each colour DMB represents a specific density (● = 1.02 g/mL; ● = 1.04 g/mL; ● = 1.06 g/mL; ● = 1.08 g/mL; ● = 1.10 g/mL; ● = 1.13 g/mL). **C.** Direct addition of phenol to Percoll resulted in aggregation due to the lowering of pH. **D.** 50% (v/v) Percoll or Percoll PLUS was prepared in PBS or 1% phenol-19% ethanol to generate self-forming gradients as described in Section 2.6.4.6, and calibrated with the use of DMBs. Resulting densities of 50% (v/v) Percoll / Percoll PLUS solutions produced in PBS and 1% phenol-19% ethanol were 1.066 g/mL and 1.043 g/mL respectively, calculated based on the equation provided in the Percoll product handbook (GE Healthcare Life Sciences).

3.2.4 Sucrose cushion centrifugation

Another approach that could facilitate the separation of *Salmonella* bacteria from macrophage debris is a sucrose cushion, a method that has previously been used successfully to isolate bacteria from nitrogen-fixing legume nodules (Ching *et al.*, 1977) and bacterial-containing phagosomes from infected macrophages (Lührmann and Haas, 2000). For separating *Salmonella* from macrophage lysate, the sucrose cushion was designed to be less dense than *Salmonella* cells, to allow bacterial cells to be pelleted during centrifugation, while keeping the unwanted cell debris in suspension. A 2.5% (w/v) sucrose cushion and four different centrifugation settings (800 x *g*, 1,600 x *g*, 2,400 x *g* and 3,200 x *g*) were first tested to identify the most appropriate centrifugation settings. As expected, centrifugation at 3,200 x *g* pelleted most bacterial cells, and a low-speed pre-spin at 800 x *g* was also found to be effective in reducing the number of intact macrophages in the pellets obtained from mixed *Salmonella*-macrophage samples (**Figure 3.6A**).

To minimise the possibility of cell debris from contaminating the *Salmonella* cell pellet, 5% (w/v) and 10% (w/v) sucrose cushions were compared using centrifugation at 3,200 x *g*. The results showed that the 5% (w/v) sucrose cushion performed better than the 10% (w/v) sucrose cushion. More bacteria pelleted at the bottom of the 50 mL centrifuge tube when the 5% (w/v) sucrose cushion was used (**Figure 3.6B**).

Experiments to determine the effectiveness of sucrose cushion centrifugation in improving the separation of *Salmonella* from infected macrophages are reported in Section 3.2.6.

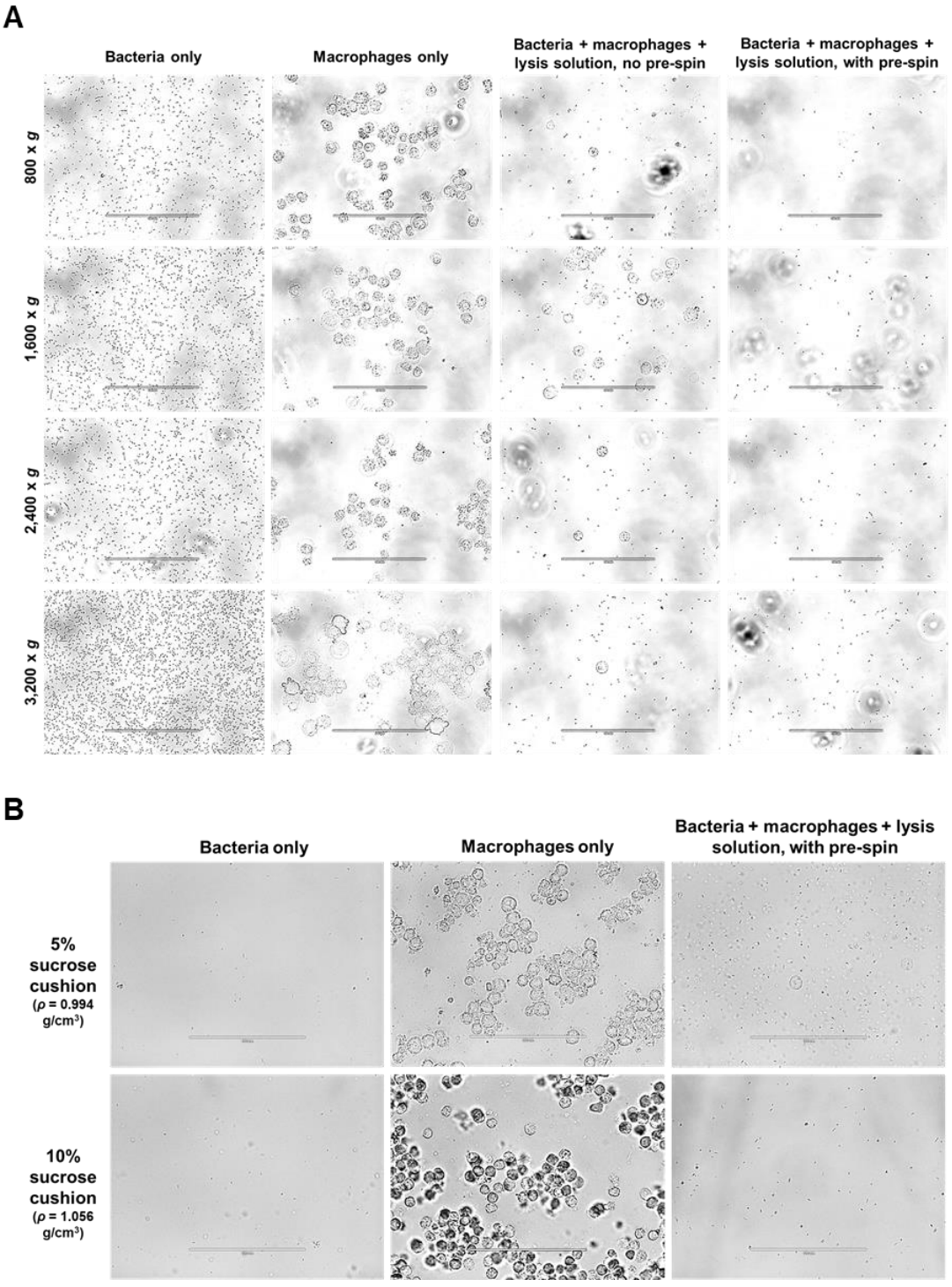

Figure 3.6 Sucrose cushion centrifugation of mixed *Salmonella*-macrophage samples

A. Determining optimal centrifugation settings on a 2.5% (w/v) sucrose cushion. **B.** Comparing the effectiveness of 5% (w/v) and 10% (w/v) sucrose cushions for allowing *Salmonella* cells to pellet following centrifugation at 3,200 x *g*, 5 min. Experimental details are described in Section 2.6.4.3. Scale bar represents 100 µm.

3.2.5 Lysate homogenisation with cell strainers

Successful RNA isolation requires both efficient cell disruption and effective lysate homogenisation from all types of samples (Rio *et al.*, 2010). Certain cell disruption methods simultaneously homogenise cell lysates, and other methods such as detergent-based disruption may require follow-up homogenisation. However, commercially available homogeniser spin columns such as QIAshredder (QIAGEN) and Omega Homogenizer Column (Omega Bio-tek) were not suitable for the larger lysate volumes produced in our intra-macrophage *Salmonella* RNA isolation protocol. Cell strainers, often used to homogenise animal tissues or to obtain single-cell suspensions, were the next best alternatives.

Cell strainers (pluriSelect) of mesh sizes ranging from 1 µm to 30 µm were first tested to determine if macrophage lysate could be filtered successfully. As expected, filtration through the larger mesh sizes was much easier and quicker, but overall initial trials suggested that even the smallest 1 µm cell strainer would homogenise a macrophage lysate. Follow-up trials to identify the most appropriate mesh size (1 µm, 5 µm or 10 µm) to exclude intact macrophages but not bacteria gave interesting observations: intact macrophages appeared in the flow-through when the 1 µm cell strainer was used (**Figure 3.7**), even though intact RAW 264.7 macrophage cells are approximately 12–16 µm in diameter. The presence of macrophages in the 1 µm cell strainer flow-through could reflect sliding of the macrophages through the mesh pores. Similar bacterial numbers in the flow-through were observed for all three strainers, and the 1 µm cell strainer was deemed to be the most suitable and used in subsequent experiments.

Experiments to determine the effectiveness of cell strainers to improve the separation of *Salmonella* from infected macrophages are reported in Section 3.2.6.

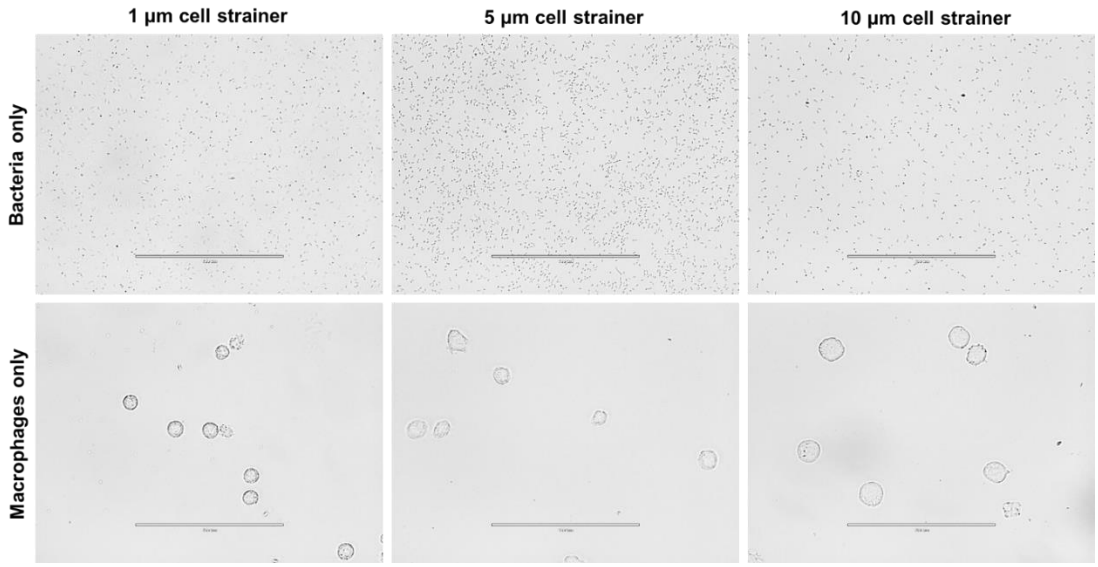

Figure 3.7 Cell strainers with 1 µm, 5 µm or 10 µm mesh size allow intact macrophages and bacteria to pass through

1 mL of ESP *S.* Typhimurium 4/74 culture or RAW 264.7 macrophage cells was applied to a cell strainer fitted to a 50 mL centrifuge tube, and allowed to filter through under gravity. A drop of the flow-through was visualised on the EVOS FL Cell Imaging System to determine the presence of bacterial or macrophage cells. Experimental details are described in Section 2.6.4.2. Scale bar represents 100 µm.

3.2.6 First evaluation of alternative methods for separation of intra-macrophage *Salmonella*

To determine the effectiveness of sucrose cushion centrifugation and cell strainer filtration in improving the separation of *Salmonella* from infected macrophages, five alternative protocols (designated Alt1 to Alt5) were designed and compared against our current protocol for preparing intra-macrophage bacterial RNA (**Figure 3.8**). The 5-min 800 x *g* pre-spin was also included as I had previously shown that this low-speed centrifugation step removed intact macrophage cells (and possibly macrophage nuclei) that remain after SDS lysis (**Figure 3.6B**). Based on the resulting RNA profiles (**Figure 3.9**), filtering the macrophage lysate through the 1 µm cell strainer alone (method Alt1) gave the best *Salmonella* (RNA) recovery (lane IV). Introducing all three steps to the existing protocol (method Alt3), or just the use of low-speed spin with or without sucrose cushion (methods Alt4 and Alt5) also improved the recovery of *Salmonella* (lanes VI, VII and VIII). Combining the pre-spin with filtration through 1 µm cell strainer (method Alt2) did reduce the amount of eukaryotic RNA (lane V), but a very low level of *Salmonella* RNA was isolated, suggesting that bacterial cells had been lost in the filtration and centrifugation steps.

Although the use of the 1 µm cell strainer improved the separation of intra-macrophage *Salmonella*, filtering large volumes of the viscous macrophage lysate through the fine mesh was extremely challenging from a practical perspective. Even with the use of new cell strainers for filtering lysates from each infected flask of macrophages, about half of the macrophage lysate was lost per extraction due to cell strainers becoming clogged. To minimise the loss, cell strainers with larger mesh sizes (20 µm and 40 µm) were tested in combination with small mesh size cell strainers (5 µm). While the larger cell strainers allowed all of the macrophage lysate to be filtered successfully and reduced the amount of eukaryotic RNA recovered, minimal *Salmonella* RNA was recovered (**Figure 3.10**).

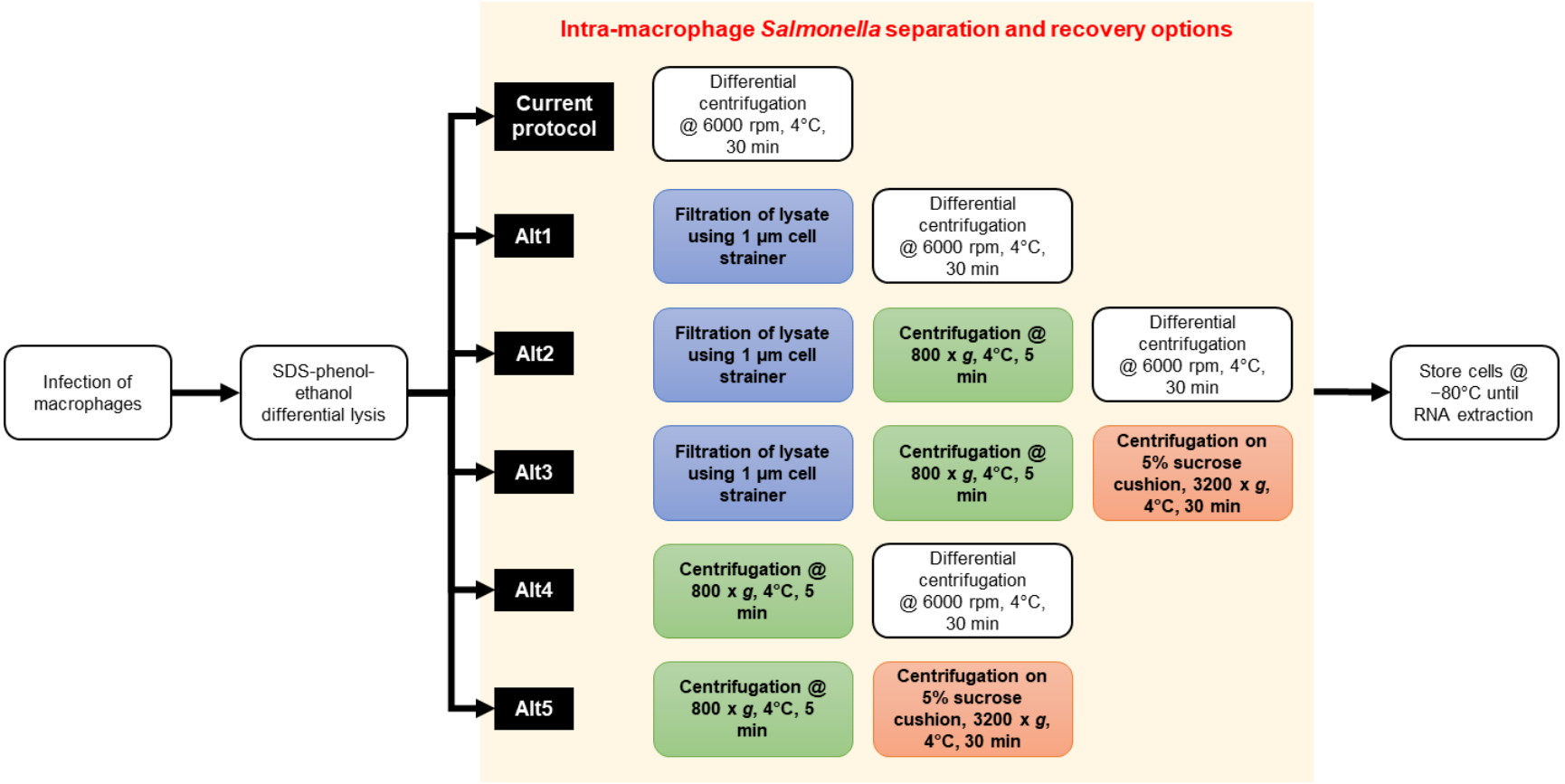

Figure 3.8 Schematic representation of the experiment comparing the alternative methods Alt1 to Alt5 with the current Hinton Laboratory protocol to separate *Salmonella* from infected macrophages

In methods Alt3 and Alt5, the 30-min centrifugation step at 6000 rpm was replaced by 30-min centrifugation on 5% (w/v) sucrose cushion. A total of 12 T75 flasks of RAW 264.7 macrophages were infected as described in Section 2.6.2 and 2 flasks were processed using each method.

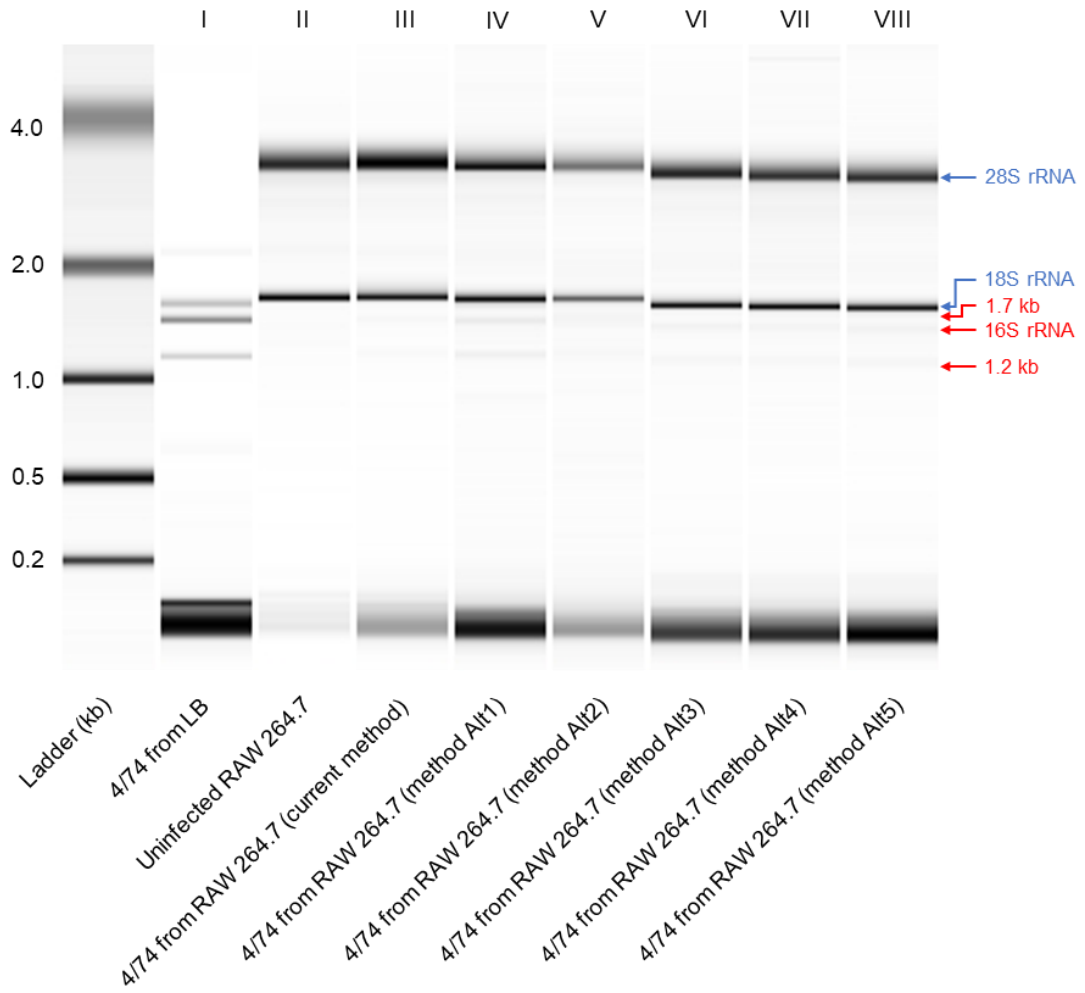

Figure 3.9 Determining the effectiveness of sucrose cushion centrifugation and cell strainer filtration in improving intra-macrophage *Salmonella* isolation

Methods Alt1 to Alt5 are explained in **Figure 3.8**. Lane I shows the locations of the three *Salmonella* rRNA bands, following extraction of *S*. Typhimurium RNA from an *in vitro* LB culture. Low levels of *Salmonella* RNA were detected using methods Alt1 (lane IV), Alt3 (lane VI), Alt4 (lane VII), Alt5 (lane) VIII, and to a lesser extent, Alt2 (lane V). Red arrows indicate the positions of the three *Salmonella* rRNA molecules. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

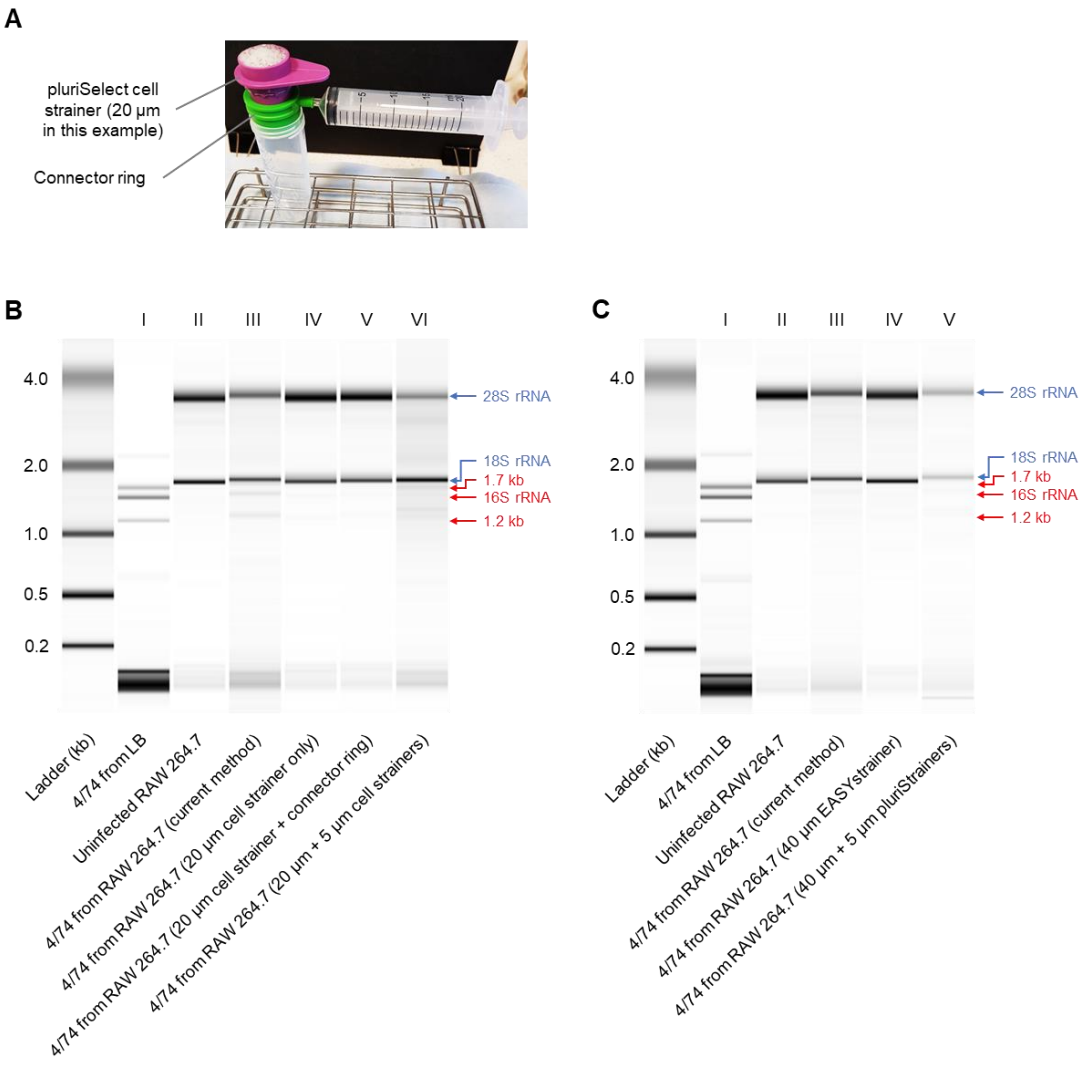

Figure 3.10 Filtration with larger mesh sized cell strainers did not improve the separation of intra-macrophage *Salmonella*

A. Set-up showing cell strainer with connector ring and attached syringe to provide low pressure filtration of macrophage lysate. Cell strainers of different mesh sizes can be stacked to provide size selective filtration. **B.** Intra-macrophage *Salmonella* RNA extraction performed using pluriSelect cell strainers, which were used directly to allow filtration by gravity (lane IV) or fitted to a connector ring attached to a 20 mL syringe (lanes V and VI) (Section 2.6.4.2). **C.** Experiments were performed using two different cell strainer brands (as labelled), with the EASYstrainer allowing filtration by gravity only. Filtration combining a larger mesh size (20 or 40 µm) and smaller mesh size (5 µm) reduced the amount of eukaryotic RNA but insufficient *Salmonella* RNA was isolated (panel **B**, lane VI; panel **C**, lane V). In panels **B** and **C**, lane I shows the locations of the three *Salmonella* rRNA bands, following extraction of *S.* Typhimurium RNA from an *in vitro* LB culture. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.2.7 Differential centrifugation using 50 mL centrifuge vs. 1.5 mL microcentrifuge tubes

The original intra-macrophage *Salmonella* RNA extraction protocol (Eriksson *et al.*, 2003) used microcentrifuge tubes and a higher centrifugation speed of 14,000 rpm for the *Salmonella* separation step. The Bioanalyzer trace of the resulting intra-macrophage *Salmonella* RNA sample, as published in the study, showed no eukaryotic RNA contamination. Given the limited success with the alternative methods tested thus far, the Eriksson *et al.* (2003) method was revisited and compared to the current version of using 50 mL centrifuge tubes for the differential centrifugation step. The RNA profiles obtained (**Figure 3.11**) suggest that more *Salmonella* RNA was recovered with the use of microcentrifuge tubes, although the problem of contaminating eukaryotic RNA remained.

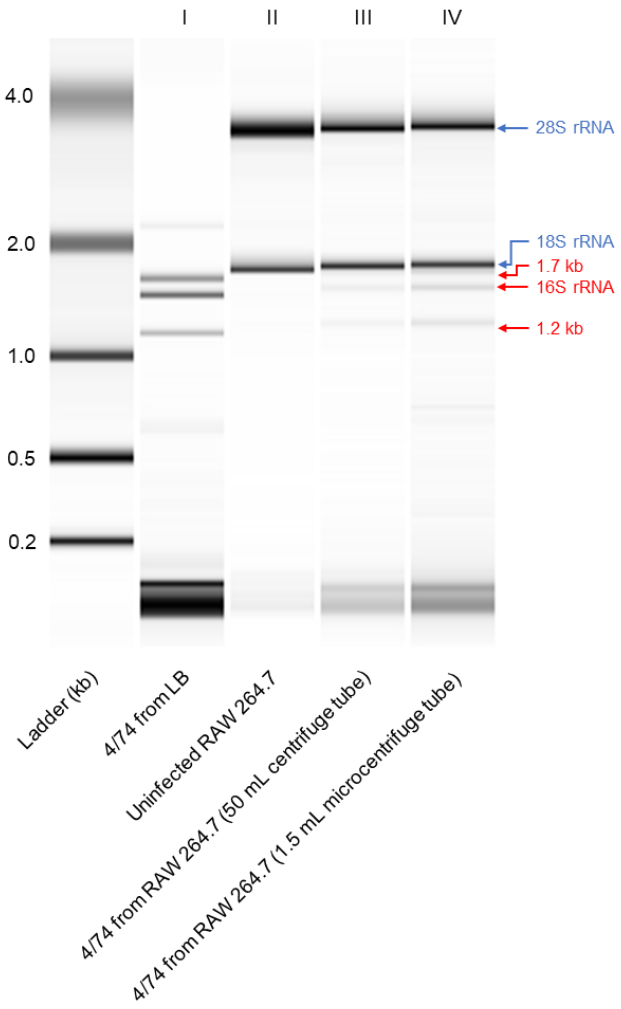

Figure 3.11 Differential centrifugation using microcentrifuge tubes improved the separation and recovery of intra-macrophage *Salmonella*

Experiment was performed as described in Section to compare the differential centrifugation method used in Eriksson *et al.* (2003) with the current protocol. Four T75 flasks of infected macrophages were processed using each method. Lane I shows the locations of the three *Salmonella* rRNA bands, following extraction of *S*. Typhimurium RNA from an *in vitro* LB culture. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.2.8 Needle and syringe homogenisation

An alternative homogenisation method involving the shearing of cell lysate through a fine-gauged needle was also assessed. Needles ranging from 21G (internal diameter 0.5 mm) to 30G (0.159 mm) were first tested to determine the most suitable needle gauge for macrophage lysate homogenisation, as a finer needle should result in greater reduction of lysate viscosity. The 21G needle was the only needle gauge that was wide enough to successfully draw up the viscous macrophage lysate.

Introducing the 21G needle and syringe homogenisation step prior to differential centrifugation of infected macrophage lysates slightly improved the recovery of *Salmonella* (**Figure 3.12B**, lane IV). Adding the 5-min low-speed spin to remove macrophage nuclei appeared to have further improved the separation of intra-macrophage *Salmonella* (**Figure 3.12C**, lanes V and VI), although *Salmonella* RNA was also detected with the current method.

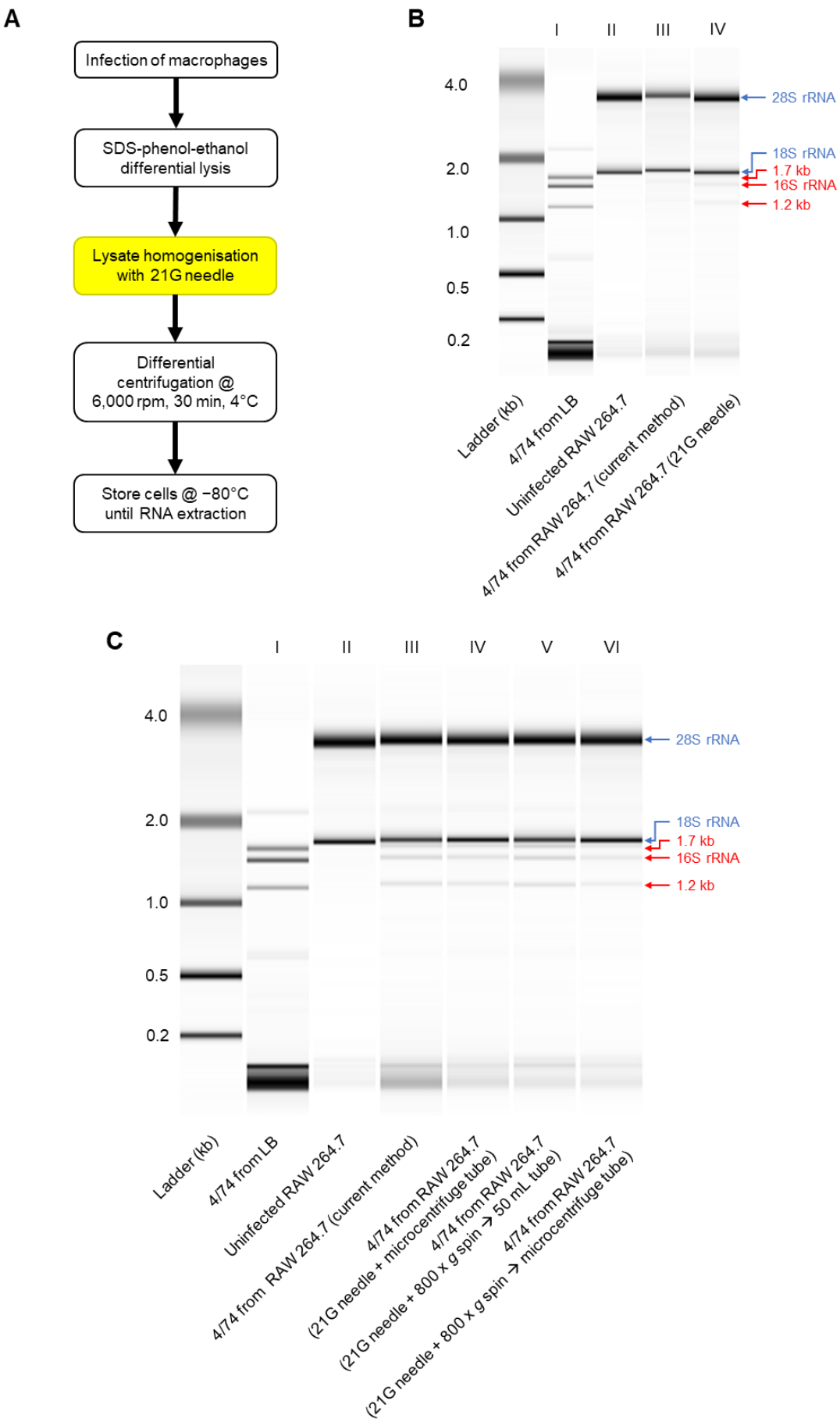
A
Infection of macrophages
SDS-phenol-ethanol differential lysis
Lysate homogenisation with 21G needle
Differential centrifugation @ 6,000 rpm, 30 min, 4°C
Store cells @ −80°C until RNA extraction
B
I
II
III
IV
4.0
2.0
1.0
0.5
0.2
28S rRNA
18S rRNA
1.7 kb
16S rRNA
1.2 kb
Ladder (kb)
4/74 from LB
Uninfected RAW 264.7
4/74 from RAW 264.7 (current method)
4/74 from RAW 264.7 (21G needle)
C
I
II
III
IV
V
VI
4.0
2.0
1.0
0.5
0.2
28S rRNA
18S rRNA
1.7 kb
16S rRNA
1.2 kb
Ladder (kb)
4/74 from LB
Uninfected RAW 264.7
4/74 from RAW 264.7 (current method)
4/74 from RAW 264.7 (21G needle + microcentrifuge tube)
4/74 from RAW 264.7 (21G needle + 800 x g spin → 50 mL tube)
4/74 from RAW 264.7 (21G needle + 800 x g spin → microcentrifuge tube)

Figure 3.12 Needle and syringe homogenisation improved the separation of intra-macrophage *Salmonella*

A. Schematic representation of the intra-macrophage *Salmonella* separation protocol, highlighting the introduction of 21G needle and syringe homogenisation step. **B.** Bioanalyzer gel-like image showing initial experiment examining the effect of 21G needle/syringe homogenisation prior to centrifugation with the current method. Lane I shows the locations of the three *Salmonella* rRNA bands, following extraction of *S*. Typhimurium RNA from an *in vitro* LB culture. Two faint bands corresponding to *Salmonella* rRNA could be seen in lane IV. Four T75 flasks of infected macrophages were processed using each method. **C.** Comparing the effect of combing 21G needle/syringe homogenisation with 5-min 800 x *g* spin and differential centrifugation in 50 mL centrifuge or 1.5 mL microcentrifuge tubes. In this extraction, all four methods gave visually similar RNA profiles that indicate the presence of *Salmonella* RNA. Two T75 flasks of infected macrophages were processed using each method. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.2.9 Combining the options: evaluating a final proposed 'improved' intra-macrophage *Salmonella* isolation protocol

All of the previous optimisation experiments in Section 3.2 involved splitting a larger batch of infected macrophages (typically eight T75 flasks) into smaller batches (either two or four flasks), each processed with a different separation method. This was done to minimise the inherent biological variability associated with infection experiments performed on different batches of macrophage cells. Considering the observations in **Figure 3.12**, it appears that the introduction of 21G needle homogenisation and a 5-min low speed spin could improve the quantity of *Salmonella* RNA isolated, regardless of the choice of 50 mL centrifuge or microcentrifuge tubes for the centrifugation step. As the concluding experiment of the optimisation of intra-macrophage *Salmonella* separation, *Salmonella* RNA was extracted from a full batch of eight T75 flasks of infected macrophages using a proposed 'improved' method (**Figure 3.13A**). However, *Salmonella* RNA was barely detected (**Figure 3.13B**) when the extraction protocol was scaled up to the original batch size.

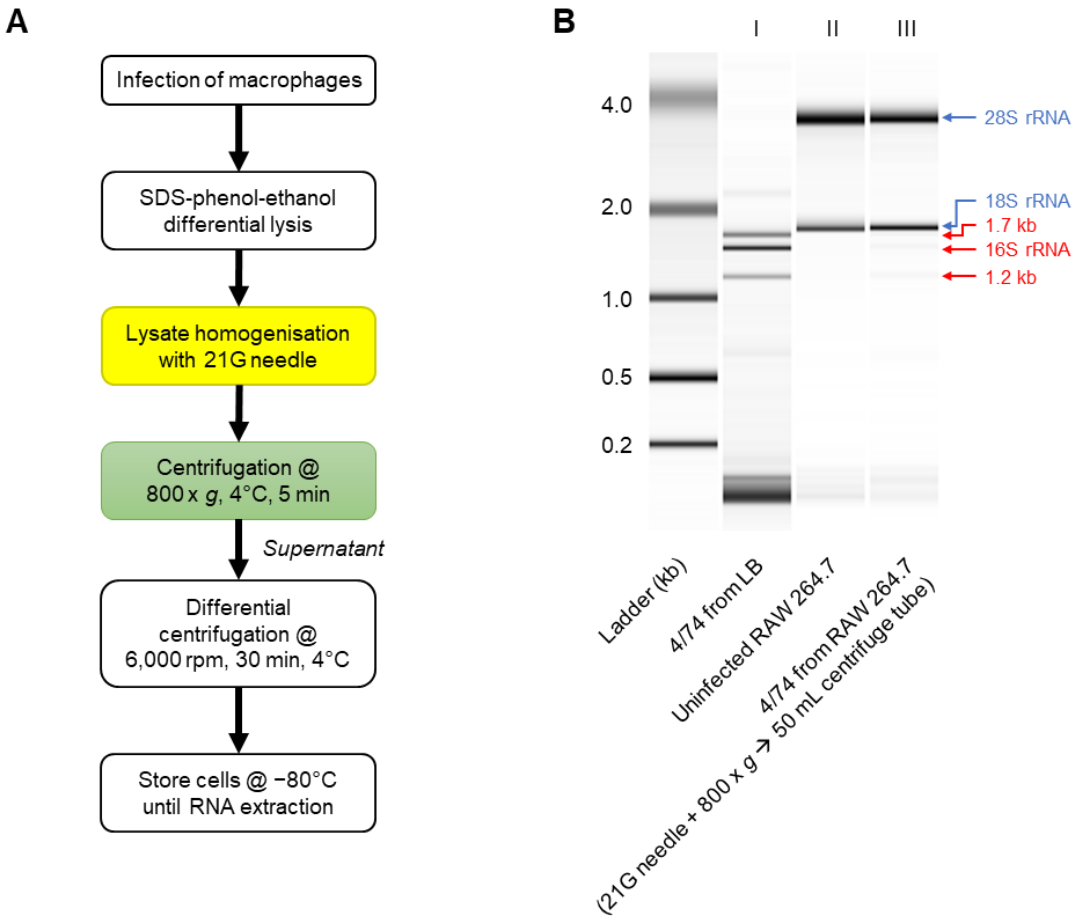

Figure 3.13 Evaluating the proposed 'improved' intra-macrophage *Salmonella* separation protocol

A. Workflow of the proposed 'improved' intra-macrophage *Salmonella* separation protocol. Steps introduced to the existing protocol (**Figure 3.1**) are highlighted in colour. **B.** Bioanalyzer gel-like image showing the RNA prepared from eight T75 flasks of *Salmonella*-infected macrophages using the proposed 'improved' method. Lane I shows the locations of the three *Salmonella* rRNA bands, following extraction of *S*. Typhimurium RNA from an *in vitro* LB culture. Blue arrows indicate the positions of the eukaryotic 28S and 18S rRNAs, and red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.2.10 Comparison of RNA purification methods

Another step in our current intra-macrophage *Salmonella* RNA extraction protocol (**Figure 3.1**) that could require optimisation is the choice of the RNA purification method or kit. Currently, all bacterial RNA purifications in the Hinton Laboratory are routinely performed using TRIzol reagent (Invitrogen). RNA yield, quality and integrity can be affected by the RNA purification method, and various studies (summarised in **Table 3.2**) have evaluated different RNA purification methods to give the best RNA sample (defined as high yield, free of contaminants, and structurally intact).

While column-based extraction methods are less time-consuming and easy to use, RNA samples prepared by column-based methods generally have poorer yields and lower quality than RNA prepared by in-solution organic methods (Martins *et al.*, 2014). This led me to compare three other in-solution RNA purification methods/kits, TRIzol Max Bacterial RNA Isolation Kit, RNAzol RT reagent, and an 'in-lab' acidic phenol-chloroform extraction protocol, against TRIzol-based RNA isolation (**Figure 3.14**).

Table 3.2 Studies evaluating different RNA purification methods or protocols

Publication	RNA purification methods compared	Organism(s) tested	Best method[a]
Jahn *et al.* (2008)	• TRIzol (Invitrogen)/RNeasy (Qiagen) • Hot SDS lysis/TRIzol/RNeasy • Hot SDS/hot phenol	*Dickeya didantii* (Gram-negative bacterium)	Hot SDS/hot phenol
Rump *et al.* (2010)	• RiboPure-Bacteria Kit (Ambion) • PureLink RNA Mini Kit (Invitrogen) • UltraClean Microbial RNA Isolation Kit (MoBio) • RNeasy Mini Kit (Qiagen) • MasterPure RNA Purification Kit (EPICENTRE Biotechnologies)	*Salmonella enterica* serovar Enteritidis strain SE5 (Gram-negative bacterium)	• No best method identified • MasterPure Kit generated highest total RNA yield but RNA sample had consideration genomic DNA contamination • UltraClean Kit isolated highly degraded RNA
Stead *et al.* (2012)	• RNA*snap*™ ("in-lab" individually designed protocol) • Catrimide/LiCl RNA isolation method • TRIzol Max Bacterial RNA Isolation Kit • RNeasy Protect Bacteria • RiboPure Bacteria Kit	*Escherichia coli* (Gram-negative bacterium) *Bacillus subtilis* (Gram-positive) *Saccharomyces cerevisiae* (eukaryotic)	RNA*snap*™
Heera *et al.* (2015)	• Phenol-free total RNA purification Kit (Amresco) • TRI Reagent (Molecular Research Centre) • RNeasy Mini Kit (Qiagen)	*Pseudomonas aeruginosa* (Gram-negative bacterium)	Phenol-free Kit
Nwokeoji *et al*. (2016)	• RNASwift ('in-lab' individually designed protocol) • TRIzol Max Bacterial RNA Isolation Kit • RiboPure Bacterial RNA extraction kit (Ambion)	*E. coli* (Gram-negative bacterium)	RNASwift

[a] The best RNA purification method was determined based on RNA yield and RIN number, except for the Nwokeoji *et al.* (2016) study, in which the Nanodrop spectrophotometric A_{260}/A_{280} ratio was used instead of the RIN number to determine RNA quality.

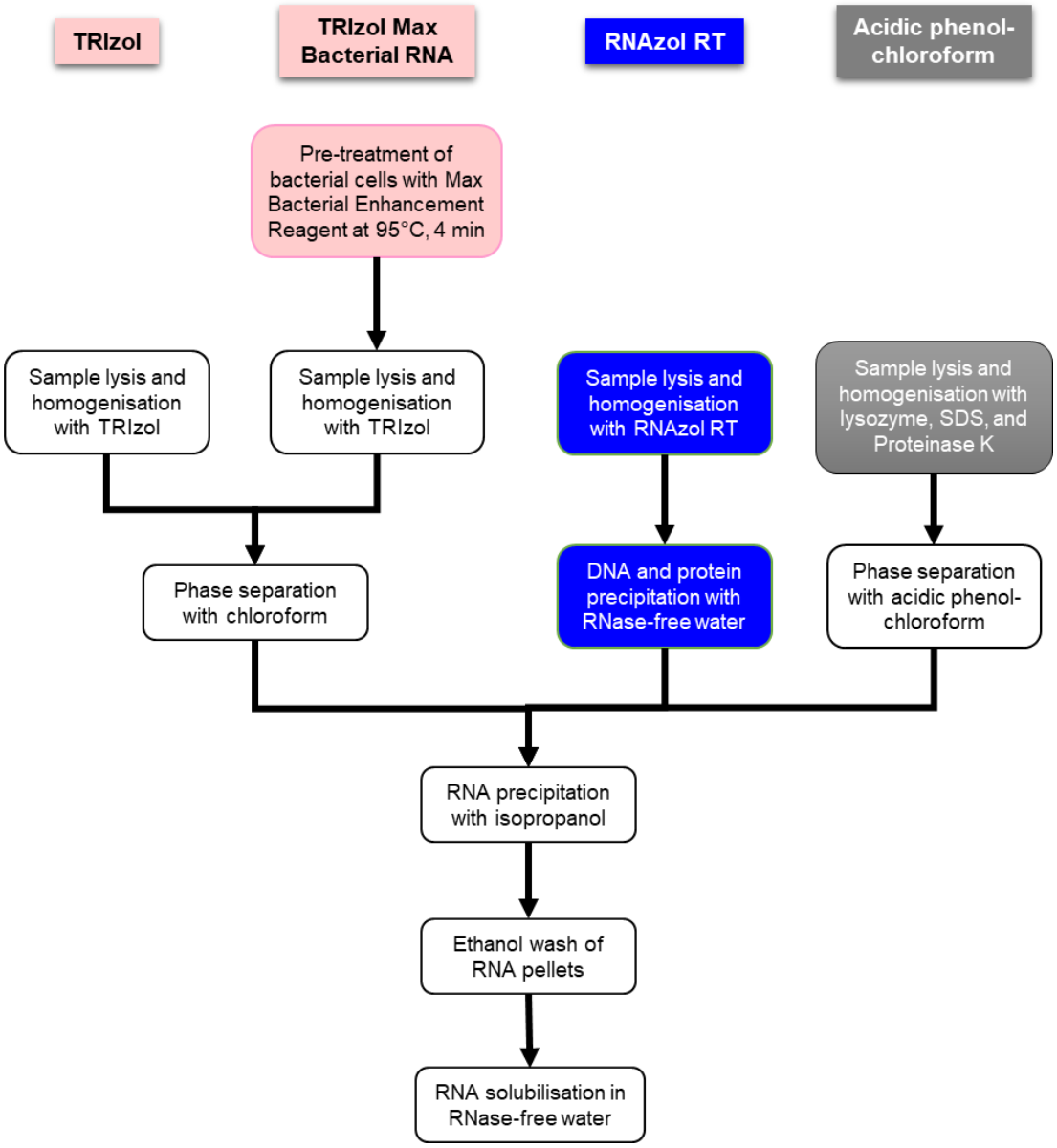

Figure 3.14 Workflow of TRIzol, TRIzol Max Bacterial RNA, RNAzol RT, and acidic phenol-chloroform RNA preparation procedures

Detailed description of each preparation procedure can be found in Section 2.6.5. Steps unique to each preparation method/kit are highlighted by different colours: pink, TRIzol-based methods; blue, RNAzol RT-based method; grey, acidic phenol-chloroform extraction

Each RNA purification method/kit was used to isolate RNA from 1 mL of 4/74 cells grown *in vitro* to ESP. RNA yield, quality and integrity were assessed as described in Section 2.6.6. While the TRIzol Max Bacterial RNA Isolation Kit gave the highest RNA yield (**Table 3.3**), most of the RNA isolated with this kit was in fact degraded (**Figure 3.15**). TRIzol and RNAzol RT yielded similar amounts of intact, high-quality *Salmonella* RNA. Acidic phenol-chloroform extraction yielded a much lower amount of RNA, although much higher RNA yields were obtained in an initial trial experiment (data not shown). Overall, there was no statistically significant differences among the different RNA preparation methods due to the large standard deviations (**Table 3.3**). Considering the yield, quality and integrity of RNA obtained, the results indicate that RNA isolation using TRIzol reagent remains the best method.

Table 3.3 Comparing *Salmonella* RNA yields from different RNA preparation methods

Method	RNA concentration[a] per 10^9 cells (ng/µL)
TRIzol	**507.5** ± 164.7
TRIzol Max Bacterial RNA Isolation Kit	**1384.6** ± 864.9
RNAzol RT	**342.1** ± 106.6
Acidic phenol-chloroform	**5.3** ± 2.7

[a] RNA concentration was determined as described in Section 2.6.6. Results show mean RNA concentrations from three biological replicates ± standard deviation.

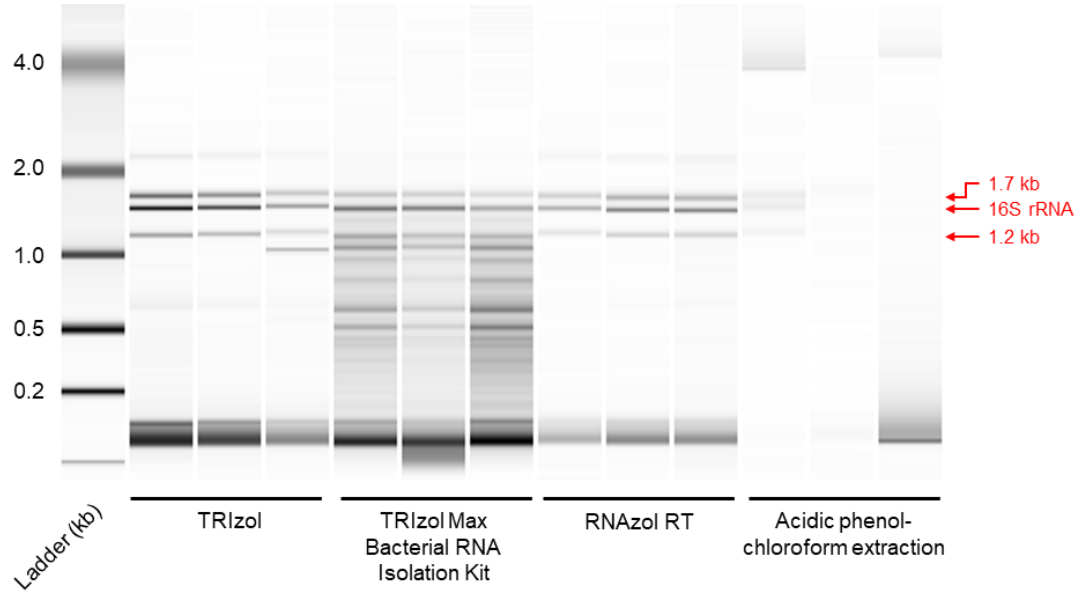

Figure 3.15 Comparing *Salmonella* RNA extracted using TRIzol, TRIzol Max Bacterial RNA, RNAzol RT, and acidic phenol-chlorofom preparation methods

Bacterial RNA was isolated from three biological replicates of 1 mL each of *S.* Typhimurium 4/74 cells grown to ESP, using the four different preparation methods as described in Section 2.6.5. Red arrows indicate the expected positions of *S.* Typhimurium rRNA, showing the 16S rRNA and at least two cleavage products of the 23S rRNA (Mattatall and Sanderson, 1996; Smith *et al.*, 1988; Winkler, 1979).

3.3 Discussion

The analysis of bacterial gene expression during infection involves many challenges, the most significant being the quantity and quality of bacterial RNA recovered. The Eriksson *et al.* (2003) study was the first report of a global transcriptional analysis from intracellular bacteria and described an important differential lysis method (**Figure 3.1**) for the stabilisation and isolation of bacterial cells from infected host cells for RNA extraction. Nevertheless, there are technical intricacies associated with this SDS-acidic phenol-ethanol based method, and experience in the Hinton Laboratory over the past 18 years has shown that the protocol did not generate reproducible results in the hands of both experienced and new users. The aim of this chapter was to improve the protocol to allow future users to reliably extract sufficient, intact intracellular *Salmonella* RNA for transcriptomic analyses.

Central to the challenge is the inefficient separation of the released bacteria from host cell debris. This can be mostly attributed to the viscosity of host cell lysates, contributed by DNA, proteins, and other biological molecules released from disrupted host cells (Newton *et al.*, 2016), and furthered confounded by the SDS detergent used in the lysis solution. It was therefore not surprising that filter-based methods to homogenise cell lysates or separate intra-macrophage *Salmonella* performed poorly, even when multiple filters were used (**Figure 3.3**, **Figure 3.9** and **Figure 3.10**).

Sigal *et al.* (2016) used a filtration-based method to isolate *Listeria monocytogenes* from infected macrophages, by capturing intracellular bacteria by filtration of the macrophage lysates. As RNase-free water was used to osmotically lyse macrophages, it is likely that the cell lysates were less viscous than SDS-based cell lysates. However, there was no RNA stabilisation of the bacterial cells prior to filtration. I consider this set-up less desirable than our method of stabilising bacterial RNA at the moment when the bacterial cells were released from the host.

The high viscosity of cell lysates is likely to explain the non-reproducibility of the proposed 'improved' bacterial separation method (**Figure 3.13**). The preceding experiments (**Figure 3.12**) suggest the use of needle syringe homogenisation combined with an initial low-speed spin to remove intact macrophages and macrophage nuclei can improve the quantity of *Salmonella* cells and therefore RNA isolated. However, these experiments involved a quarter or half the volume of infected macrophages to be processed for bacterial purification, as compared to a full-scale infection for RNA extraction intended for RNA-seq. A possible solution is to isolate *Salmonella* RNA from a smaller volume of infected macrophages then pool RNA the samples. However, this would also increase the amount of eukaryotic RNA accordingly, since none of the alternative methods tested in this chapter gave a *Salmonella* RNA sample that was free of eukaryotic RNA contamination.

To circumvent the issue of viscosity contributed by SDS, one could consider using mechanical cell lysis methods such as bead beating or homogenisation with a Dounce tissue grinder. Maintaining the presence of acidic phenol-ethanol solution throughout the lysis process should ensure bacterial RNA stability. However, using a brand-new lysis method would require extensive optimisation to ensure that host cells are lysed efficiently while leaving bacterial cells intact. Given the time constraints on the PhD project, we chose to focus on refining the tried-and-tested components of our existing protocol with minor changes.

Compared to differential centrifugation, density gradient centrifugation should improve bacterial separation from host components, because particles should settle along the supporting column of density gradient media at their respective buoyant densities (Rickwood, 2001). I attempted continuous gradient centrifugation using Percoll, and discontinuous gradient centrifugation using sucrose. Sucrose cushion centrifugation would be the easier of the two options from an operational point of view,

but it only provided a slight improvement in the quantity of *Salmonella* RNA (**Figure 3.9**). For Percoll gradient centrifugation, I expected and observed changes in Percoll properties (**Figure 3.5**) due to the presence of acidic phenol-ethanol, suggesting that our protocol set-up was not easily compatible with most commercially available reagents. Further optimisation will be required which was again not feasible in the time available.

I also considered the possibility of a better *in vitro* RNA purification method, even though TRIzol RNA extraction is widely accepted as the 'gold-standard'. Observations from the comparison of four different extraction methods/kits were generally consistent with expectations (**Figure 3.15**): TRIzol and RNAzol RT are similar reagents made up of a monophasic solution of phenol and guanidinium isothiocyanate and therefore expected to perform similarly in RNA preparation; the choice of which reagent would depend on whether chloroform phase separation needs to be avoided. RNA preparation using the 'in-lab' acidic phenol-chloroform extraction gave much lower RNA yields and could be due to incomplete lysis of bacterial cells, as earlier trials using fewer bacterial cells gave considerably higher RNA yields (data not shown). The most intriguing observation from this experiment was the highly degraded RNA that was generated with the TRIzol Max Bacterial RNA Isolation Kit; a similar observation was reported by Nwokeoji *et al*. (2016). The only possible explanation is the denaturing of RNA during the pre-treatment step, which is counterintuitive considering the Max Bacterial RNA Enhancement Reagent, as advertised by the manufacturer, should serve to protect and increase the yields of intact RNA.

Having established that TRIzol RNA extraction remains the best method, one addition worth considering is the use of a coprecipitant (such as GlycoBlue™) during bacterial RNA isolation. Although all RNA preparation protocols mention an RNA 'pellet'

following the alcohol precipitation step, in reality the pellet is often invisible, especially when dealing with minute amounts of bacterial RNA yield from an intracellular extraction protocol. Coprecipitants are inert substances used to aid recovery of nucleic acids during alcohol precipitations and can aid in visualisation of the pelleted precipitate after centrifugation to prevent pellet loss.

Finally, a major limitation in the design of the optimisation experiments described in this chapter was the lack of a quantitative measure to compare the efficiency of the methods (that is, intra-macrophage *Salmonella* RNA yield relative to the host RNA). Analysis of RNA on the Bioanalyzer instrument is indispensable to verifying the integrity and purity of recovered *Salmonella* RNA, but only confirms at best the presence or absence of RNA. Qubit measurements of bacterial RNA recovered from infected cells would not be reliable, as the concentrations could be biased by the presence of contaminating host RNA. Instead, a quantitative PCR method such as that described in Westermann *et al.* (2016) could be used to estimate the proportions of *Salmonella* and macrophage RNA in our RNA preparations. In fact, these quantitative PCR experiments were initiated but were halted and eventually cancelled due to the COVID-19 pandemic-related closure of university and research labs.

In summary, findings from this chapter reinforce the technical challenge and inherent variability associated with our SDS-phenol-ethanol based differential lysis approach to isolating intra-macrophage *Salmonella* RNA. I showed that filter-based methods were not suitable for working with viscous cell lysates, and density gradient centrifugation provided little to no improvement. Although syringe needle homogenisation and prior-removal of macrophage nuclei with low-speed centrifugation showed promise for improving the recovery of intra-macrophage *Salmonella* RNA, these adjustments did not reliably increase the bacterial RNA yield. Dual RNA-seq methods could avoid altogether the hurdle of physical separation of

pathogen and host. However, dual RNA-seq is a particularly expensive option due to the extremely high sequence depth that is required, and involves a different set of challenges to be considered (reviewed in Westermann *et al*., 2017).

While I was unable to arrive at a fool-proof optimised method, our differential lysis approach will likely continue to be a valuable technique to other researchers who are interested in intracellular pathogen transcriptomics, and the findings presented here may inspire further individual customisation of the protocol to suit particular requirements.

Chapter 4 :

Genetic requirements of African and Global Epidemic *Salmonella* Enteritidis for growth *in vitro*

4.1 Introduction

The African *S.* Enteritidis strain D7795 and the Global Epidemic strain P125109 share extensive synteny and collinearity and are separated by ~1000 SNP differences in their whole genome nucleotide sequences (**Figure 1.2**) (Feasey *et al.*, 2016; Perez-Sepulveda *et al.*, 2021). However, these two bacterial strains are associated with distinct disease syndromes, with P125109 causing gastrointestinal disease while D7795 is associated with invasive bloodstream infection in sub-Saharan Africa (Feasey *et al.*, 2016). The availability of whole genome sequences of both P125109 and D7795 has made it possible to use high-throughput techniques to investigate the genetic basis for the observed clinical differences.

Following the conclusion of Chapter 3, which explained that performing transcriptomic studies of *S.* Enteritidis during macrophage infection would be a challenging endeavour with no guaranteed returns within the timespan of this PhD project, a different technique involving random transposon insertional mutagenesis and high-throughput sequencing was chosen to characterise the gene functions of the two *S.* Enteritidis genomes in infection-relevant conditions. This transposon insertion sequencing (TIS) technique, also termed Tn-Seq or TraDIS in the literature (introduced in Chapter 1), involves the construction of a library of transposon mutants in the strain of interest, followed by challenges with different environmental conditions. The relative changes in abundance of each transposon mutant before and after the treatment reflects the contribution of each gene to survival and adaptation to a particular environment.

TIS is a powerful technique for identifying required genes, which are genes critical for survival and growth of an organism (Rancati *et al.*, 2018). Studies on *S.* Typhimurium, *S.* Typhi, *E. coli* and *Pseudomonas aeruginosa* revealed that gene essentiality differs between strains of the same bacterial species, varying with genetic backgrounds,

ecological niches and lifestyles (Barquist *et al.*, 2013b; Canals *et al.*, 2012; Langridge *et al.*, 2009; Poulsen *et al.*, 2019; Rousset *et al.*, 2021). TIS has also been used to investigate conditional gene requirements, which are genes required for growth under specific environmental or nutritional conditions (Barquist *et al.*, 2013a); such conditional gene requirements have been reported for *S.* Typhimurium (Canals *et al.*, 2019a; Karash and Kwon, 2018; Khatiwara *et al.*, 2012) and *S.* Typhi (Kingsley *et al.*, 2018; Langridge *et al.*, 2009). In contrast, the genetic requirements of *S*. Enteritidis remain to be identified. Knowledge of required and conditional gene requirements for a common bacterial pathogen like *S.* Enteritidis is useful, because apart from expanding our understanding about the basic biology of the pathogen, the genetic requirements may have applications in novel therapeutics development (Juhas *et al.*, 2012).

In this chapter I report the TIS-based identification of the genetic requirements of *S.* Enteritidis P125109 and D7795 for survival and growth in three different *in vitro* growth conditions. The overall experimental strategy is presented in **Figure 4.1**. As mentioned in Chapter 3, as a consequence of the COVID-19 pandemic and the associated closure of universities in March 2020, I was unable to perform experimental validation of phenotypes reported in this chapter.

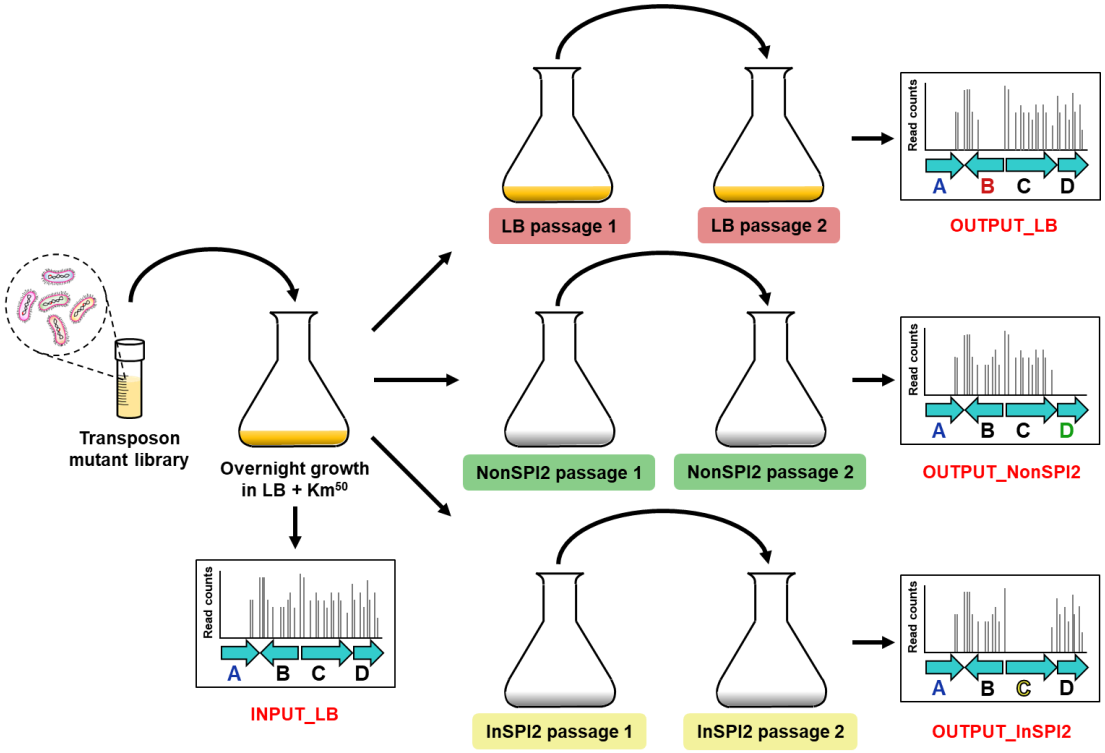

Figure 4.1 Schematic representation of transposon insertion sequencing (TIS) screen of *S.* Enteritidis transposon libraries in LB, NonSPI2 and InSPI2 media

Experimental details are provided in Section 2.7.2, and recipes for LB, NonSPI2 and InSPI2 media are available in **Table 2.1**. Genomic DNA from the input and output samples was purified and sequenced by Illumina technology. The sequence reads were mapped to the P125109 or D7795 reference genome to identify unique transposon insertion sites. The genes (*A*, *B*, *C* or *D*) highlighted in colour illustrate how required genes for a particular environmental condition were identified. We used the number of transposon insertions per gene and gene length to derive an insertion index (see text in Section 4.2.1).

4.2 Results

4.2.1 Profiling of *S.* Enteritidis P125109 and D7795 transposon libraries

Transposon libraries were constructed in strains P125109 and D7795 as described in Section 2.7.1. Each pool of transposon mutants was grown in LB (input) and passaged two times successively at 37°C in three different growth media: a nutrient rich medium, LB (output_LB), an acidic phosphate-limiting PCN-based minimal media that induces SPI-2 expression, designated InSPI2 (output_InSPI2), and a neutral pH PCN-based minimal media that does not induce SPI-2 expression, designated NonSPI2 (output_NonSPI2) (Löber *et al.*, 2006) (**Figure 4.1**). Genomic DNA from the input and output samples was purified and prepared for Illumina sequencing of DNA adjacent to the transposon, as described in Section 2.7.4. Sequencing was performed on two lanes of an Illumina NovaSeq6000 system, generating a total of 2,096,901,692 reads (523,450,580 read pairs from the first lane and 525,000,266 read pairs from the second). The sequencing data was processed as described in Section 2.8.4. **Table 4.2** shows the number of sequence reads obtained, the sequenced reads that contained the transposon tag sequence, and the sequence reads that were mapped to the respective *S.* Enteritidis genomes.

Sequence analyses of the input libraries identified 246,743 unique transposon insertion sites in P125109 and 195,646 unique insertions in D7795, an average of one insertion per 19 nucleotides in the P125109 genome and one insertion per 24 nucleotides in the D7795 genome. The complete data sets are available for visualisation in two online JBrowse genome browsers for the respective strains at: **hintonlab.com/jbrowse/index.html?data=tradis_P125109/data** and **hintonlab.com/jbrowse/index.html?data=tradis_D7795/data**. The browsers show the transposon insertion profiles of the chromosome and plasmid(s) in the context of the full annotation of the coding genes and non-coding RNA genes of P125109 and D7795, which were obtained by a comparative genomic approach based on

annotated genes for *S.* Typhimurium ST19 (Kröger *et al.*, 2012) and *S.* Typhimurium ST313 (Canals *et al.*, 2019b) (Section 2.8.1; also see Perez-Sepulveda *et al.*, 2021).

An insertion index was calculated for each gene by dividing the number of unique insertions for any given gene by its gene length, and essentiality analysis was performed as described in Section 2.8.4, classifying genes as “required, “not required” and “ambiguous”. “Required” genes in this study included genes essential for bacterial viability (i.e. genes that when disrupted lead to irreversible growth arrest or cell death), and genes with a strong contribution to fitness in a particular environmental condition (Rancati *et al.*, 2018) (**Table 4.1**). Genes shorter than 200 nt in length could not be robustly analysed and were called “short”. The number of reads, transposon insertion sites, insertion index, and the essentiality calls per gene for all conditions tested are summarised in **Appendix 2** (for P125109) and **Appendix 3** (for D7795).

Table 4.1 Definition of “required” genes referred to in each growth condition

Condition	Definition of “required”
Input	Genes essential for bacterial viability (I.e. genes when disrupted lead to irreversible growth arrest or cell death) plus genes required for survival and growth after a single passage in LB (see transposon library construction details in Section 2.7.1)
LB output	Required genes in input plus genes required for optimal growth in LB
NonSPI2 output	Required genes in input plus genes required for optimal growth in NonSPI2
InSPI2 output	Required genes in input plus genes required for optimal growth in InSPI2

Table 4.2 Transposon insertion sequencing (TIS) data set derived from *S.* Enteritidis strains P125109 & D7795 transposon libraries assayed in different environmental growth conditions

Sample	De-multiplexed reads with transposon (Tn) tag	Reads mapped to genome (%)	Deduplicated reads	Deduplicated reads with *Salmonella* sequence next to Tn tag	Reads mapped to annotated features (%)	Unique insertions[a]	Unique insertions in annotated features (%)
P125109_input	10,116,096	9,655,444 **(95.5)**	4,987,810	4,477,826	4,035,228 **(90.1)**		
P125109_input_1	13,181,152	12,451,393 **(94.5)**	5,929,503	5,097,132	4,605,547 **(90.4)**		
P125109_input_2	9,124,041	8,766,407 **(96.1)**	4,188,729	3,802,133	3,441,397 **(90.5)**	246,743	198,848 **(80.6)**
P125109_input_3	10,882,707	10,326,133 **(94.9)**	5,235,438	4,649,938	4,180,477 **(90.0)**		
P125109_input_4	11,297,748	10,872,306 **(96.2)**	5,005,975	4,518,348	4,058,360 **(89.8)**		
P125109_output_LB_1	12,030,707	11,651,597 **(96.9)**	4,021,559	3,579,109	3,197,843 **(89.4)**	169,994	136,091 **(80.0)**
P125109_output_LB_2	16,698	4,755 **(28.5)**	16,533	3,080	2,802 **(91.0)**		
P125109_output_NonSPI2_1	5,121,905	4,439,144 **(86.7)**	2,837,223	2,135,751	1,941,434 **(90.9)**	179,066	143,428 **(80.1)**
P125109_output_NonSPI2_2	9,370,825	9,037,650 **(96.4)**	4,043,876	3,675,457	3,295,540 **(89.7)**		
P125109_output_InSPI2_3	4,436,371	4,117,125 **(92.8)**	2,434,325	2,073,730	1,887,756 **(91.0)**	183,071	147,380 **(80.5)**
P125109_output_InSPI2_4	5,522,395	5,132,723 **(92.9)**	2,898,359	2,453,047	2,225,620 **(90.7)**		

Table 4.2 *(continued)* TIS data set derived from *S.* Enteritidis strains P125109 & D7795 transposon libraries assayed in different environmental growth conditions

Sample	De-multiplexed reads with transposon (Tn) tag	Reads mapped to genome (%)	Deduplicated reads	Deduplicated reads with *Salmonella* sequence next to Tn tag	Reads mapped to annotated features (%)	Unique insertions[a]	Unique insertions in annotated features (%)
D7795_input	5,810,812	5,227,124 **(90.0)**	3,324,042	2,684,000	2,380,685 **(88.7)**		
D7795_input_1	4,308,209	4,125,342 **(95.8)**	2,596,669	2,391,061	2,137,330 **(89.4)**		
D7795_input_2	4,681,961	4,469,623 **(95.5)**	2,919,407	2,683,719	2,404,285 **(89.6)**	195,646	156,928 **(80.2)**
D7795_input_3	6,924,673	6,519,532 **(94.2)**	4,035,273	3,593,136	3,217,400 **(89.5)**		
D7795_input_4	5,591,764	5,220,325 **(93.4)**	3,192,096	2,776,353	2,453,941 **(88.4)**		
D7795_output_LB_1	6,092,101	5,349,326 **(87.9)**	3,680,453	2,872,672	2,537,539 **(88.3)**	152,287	121,849 **(80.0)**
D7795_output_LB_2	5,854,093	5,517,476 **(94.6)**	2,917,239	2,545,299	2,261,569 **(88.9)**		
D7795_output_NonSPI2_3	5,392,936	5,158,607 **(95.7)**	506,977	236,366	209,492 **(88.6)**	89,304	71,533 **(80.1)**
D7795_output_NonSPI2_4	4,689,483	4,491,646 **(95.8)**	403,402	191,253	169,396 **(88.6)**		
D7795_output_InSPI2_3	4,749,169	4,567,659 **(96.2)**	540,841	338,751	303,626 **(89.6)**	93,573	74,866 **(80.0)**
D7795_output_InSPI2_4	4,250,129	4,111,972 **(96.8)**	398,803	249,904	223,381 **(89.4)**		

[a] Number of unique insertions for each strain and condition was calculated by combining reads from the appropriate biological replicates.

4.2.2 Genetic requirements of P125109

4.2.2.1 Identification of genes required for *in vitro* growth

Essentiality analysis of the P125109 input library identified 497 required genes, 3516 dispensable genes and 317 ambiguous genes, and 693 genes were classified as short (**Figure 4.2A**). Of the 497 required genes (**Appendix 2**), 492 genes were located on the chromosome and 5 genes on the pSENV virulence plasmid (*traJ*, *samA*, *samB*, *SEN_p0037* and *SEN_p0021*).

To provide a functional context for the required genes, the distribution of Cluster of Orthologous Groups (COG) functional categories across the genome was determined. As we recently updated the annotation of the P125109 reference genome following PacBio sequencing of the strain used in-house (Perez-Sepulveda *et al.*, 2021), the COG categories were re-assigned using eggNOG-mapper v2 (Huerta-Cepas *et al.*, 2017; Huerta-Cepas *et al.*, 2019) (Section 2.8.2), which uses only 20 of the 26 functional categories used by the NCBI COG database (**Table 4.3**).

Based on the COG classifications, the majority of the required genes were involved in translation (J, 17%) and cell wall biogenesis (M, 5%), followed by an approximately equal distribution in the categories of transcription (K, 3%), replication (L, 3%), energy production (C, 3%) and various metabolic processes (E, F, H and I) (**Figure 4.2B** and **Table 4.4**). Ninety-six genes (16%) were classified in the “Poorly Characterised” category, including 28 genes belonging to Regions of Difference (RODs) (Thomson *et al.*, 2008), prophage regions and SPIs, highlighting the limitations associated with the eggNOG mapper tool and COG classifications in general. Eleven genes associated with SPIs were identified as required: *SEN0277* in SPI-6, several *ssa* and *ttr* genes in SPI-2, and *SEN4250* in SPI-10.

Table 4.3 Cluster of Orthologous Genes (COGs) categories assigned by eggNOG mapper

	COG	Description	Example functions/proteins and associated genes
Information Storage and Processing	J	Translation, ribosomal structure and biogenesis	tRNA-synthetases: *glnS*, *proS*, *ileS*, *leuS* Ribosome components: *rpl* genes, *rpm* genes, *rps* genes Initiation, elongation, and peptide chain release factors: *infA*, *efp*, *prfAH*, *pth* RNA chaperones: *proQ*, *hfq*
	A	RNA processing and modification	RNA 3'-terminal phosphate cyclase: *rtcA* Oligoribonuclease (3'-5' exoribonuclease): *orn*
	K	Transcription	RNA polymerase: *rpoABC* Sigma factors: *rpoESD* Anti- and termination factors: *nusBAG* Initiation, elongation, termination factors Transcriptional regulators: *hns*, *fnr*, *hilA* Nucleoid-associated proteins: *hns* Response regulators of two-component systems (TCS): *phoP* Cold shock proteins *csp*
	L	Replication, recombination and repair	Primosome-associated: *dnaGAC*, *priABC*, *rep*, *ssb* DNA polymerase III: *dnaEN*, *holABCE* DNA polymerase II: *polB* DNA polymerase I: *polA* Gyrase and topoisomerases: *gyrAB*, *parCE* Endo- and exonucleases: *mutHLMT*, *rnh*, *ruvAC*, *uvrABCD* Transposase, integrase
Cellular Processes and Signalling	D	Cell cycle control, cell division, chromosome partitioning	Cell division proteins: *damX*, *ftsAEUKLNQWXYZ*, *sulA* Chromosome partitioning: *mukBEF* Cell partitioning: *minCD*
	V	Defense mechanisms	Transporters and efflux pumps: *sapBC*, *acrDEF* Restriction enzymes: *hsdMRS* Beta-lactam-associated: *ampEDH*
	T	Signal transduction mechanisms	Sensor kinases of TCS: *envZ*, *phoQ*, *arcB*, *baeS*, *ssrA*
	M	Cell wall / membrane / envelope biogenesis	Peptidoglycan synthesis: *murABCDEFGI*, *mraY* Lipopolysaccharide biosynthesis: *rfaBCFGIJKLQ* O-antigen biosynthesis: *rfb* genes Colanic acid polysaccharide capsule biosynthetic genes: *wca* genes
	N	Cell motility	Fimbrial proteins: *bcfDEFG*, *fim*ADFHI, *pegABD, stbACD* Flagella biosynthesis & components: *flgABCDEFGHIJKLMN*, *flhABE*, *fliCDEFGH*, *motAB* SPI secretion system apparatus proteins: *spaOS* (SPI-1), *ssaCKNQ* (SPI-2)
	W	Extracellular structures	Autotransporter adhesin: *sadA* Outer membrane usher protein *fimD*
	U	Intracellular trafficking, secretion, and vesicular transport	Sec translocase SPI-1 TTSS (*spaPQR*) SPI-2 TTSS (*ssaJRSTUV*)

Table 4.3 (*continued)* Cluster of Orthologous Genes (COGs) categories assigned by eggNOG mapper

	COG	Description	Example functions/proteins and associated genes
Cellular Processes and Signalling	O	Post-translational modification, protein turnover, chaperones	*ccm* genes *clpABPX* Molecular chaperones: *dnaAK* Fe-S cluster: *sufBCD* Lon protease: *lon* *ppiBAC*
Metabolism	C	Energy production and conversion	ATP synthase: *atpBEFGH* Cytochrome: *cybBC*, *cydABCD* *nuo* genes
	G	Carbohydrate transport and metabolism	Galactose metabolism and transport: *galKMPT* Galactarate metabolism and transport: *garDKL* Mannose metabolism and transport: *manAXYZ*
	E	Amino acid transport and metabolism	Arginine biosynthesis: *argACDEH*, Aromatic amino acids biosynthesis: *aroABCDEF*
	F	Nucleotide transport and metabolism	Purine metabolism: *purABCDEFGH*
	H	Coenzyme transport and metabolism	Heme biosynthesis: *hem* genes Aspartate pathway: *nadABCDE* *ribABDEF* genes
	I	Lipid transport and metabolism	*accABCD* Fatty acid biosynthesis: *fabABDFGH*
	P	Inorganic ion transport and metabolism	*fhu* genes Catalase genes*: kat* genes Magnesium transport: *mgtABC*
	Q	Secondary metabolites biosynthesis, transport and catabolism	Siderophore (enterobactin) synthesis: *entBCDEF* genes *pduACEJKLNTX* (although some are also classified under category C)
Poorly Characterised	S	Function unknown	Includes many genes associated with Regions of Difference (RODs) (Thomson *et al.*, 2008), prophage regions and SPIs

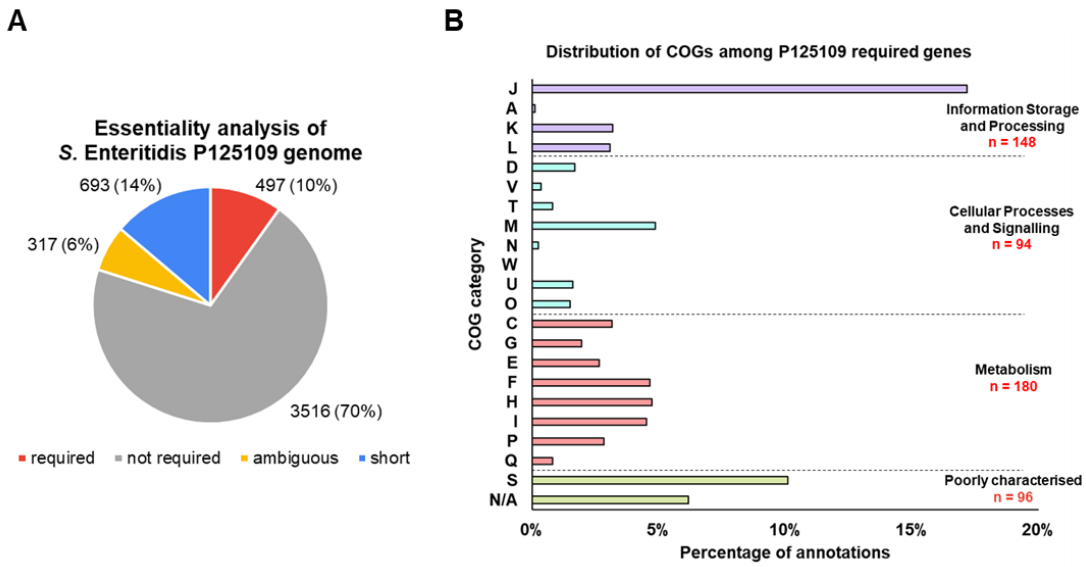

Figure 4.2 Essentiality analysis of *S.* Enteritidis P125109 genome and distribution of functional categories

A. Pie chart showing distribution of required, dispensable (not required), ambiguous and short categories of genes. **B.** Bar chart showing the distribution of the 497 P125109 required genes across the COG categories, as percentage of each category out of the total annotations assigned to required genes. The numbers in red indicate the total numerical counts of COG annotations in each category. Definitions of the COG categories are given in **Table 4.3**. An "N/A" category was added to group all genes that were not assigned a COG category by eggNOG-mapper.

4.2.2.2 Genetic requirements for optimal growth in LB, NonSPI2 and InSPI2

The P125109 transposon library was passaged twice in three different growth media under laboratory conditions: LB, NonSPI2 and InSPI2. The two passages of the transposon mutants in nutrient-rich LB allowed the identification of genes that are "advantageous" for growth (Langridge *et al.*, 2009), which are genes that promote optimal growth but are usually not required for growth in LB. The InSPI2 minimal medium partially mimics the conditions encountered by *Salmonella* in the macrophage, and stimulates expression of SPI-2 genes (Löber *et al.*, 2006). The NonSPI2 medium is a neutral pH, high-phosphate version of the InSPI2 minimal medium, the ideal comparator for InSPI2 that does not induce SPI2 expression (Löber *et al.*, 2006). The use of both media allowed the identification of genes required by P125109 to grow optimally in a synthetic defined environment, as well as providing insights for genes that may be required for optimal survival in the macrophage (further explored in Chapter 5). Genes required for growth in each media were identified by the insertion index and essentiality analysis, as described in Section 2.8.4.

Following two passages in LB, 538 genes were designated as required, which included genes that were required in the input as well as genes that contributed to fitness for *in vitro* growth in LB: 531 genes in the chromosome and 7 genes in the pSENV plasmid. A total of 565 genes were required for optimal growth after two passages in NonSPI2: 561 genes in the chromosome and 4 genes in the pSENV plasmid. Analysis of the InSPI2 output library identified 629 genes, with 622 genes located in the chromosome and 7 genes in the pSENV plasmid (**Appendix 2**).

The required gene lists for input, LB output, NonSPI2 output and InSPI2 were overlapped to identify genes shared between all conditions and genes that are only required under specific conditions. I identified 19 genes for LB, 14 genes for NonSPI2, and 48 genes for InSPI2 that were required only in the specific media; these genes were categorised as LB-only, NonSPI2-only and InSPI2-only, respectively (**Figure 4.3A**, **Appendix 4**).

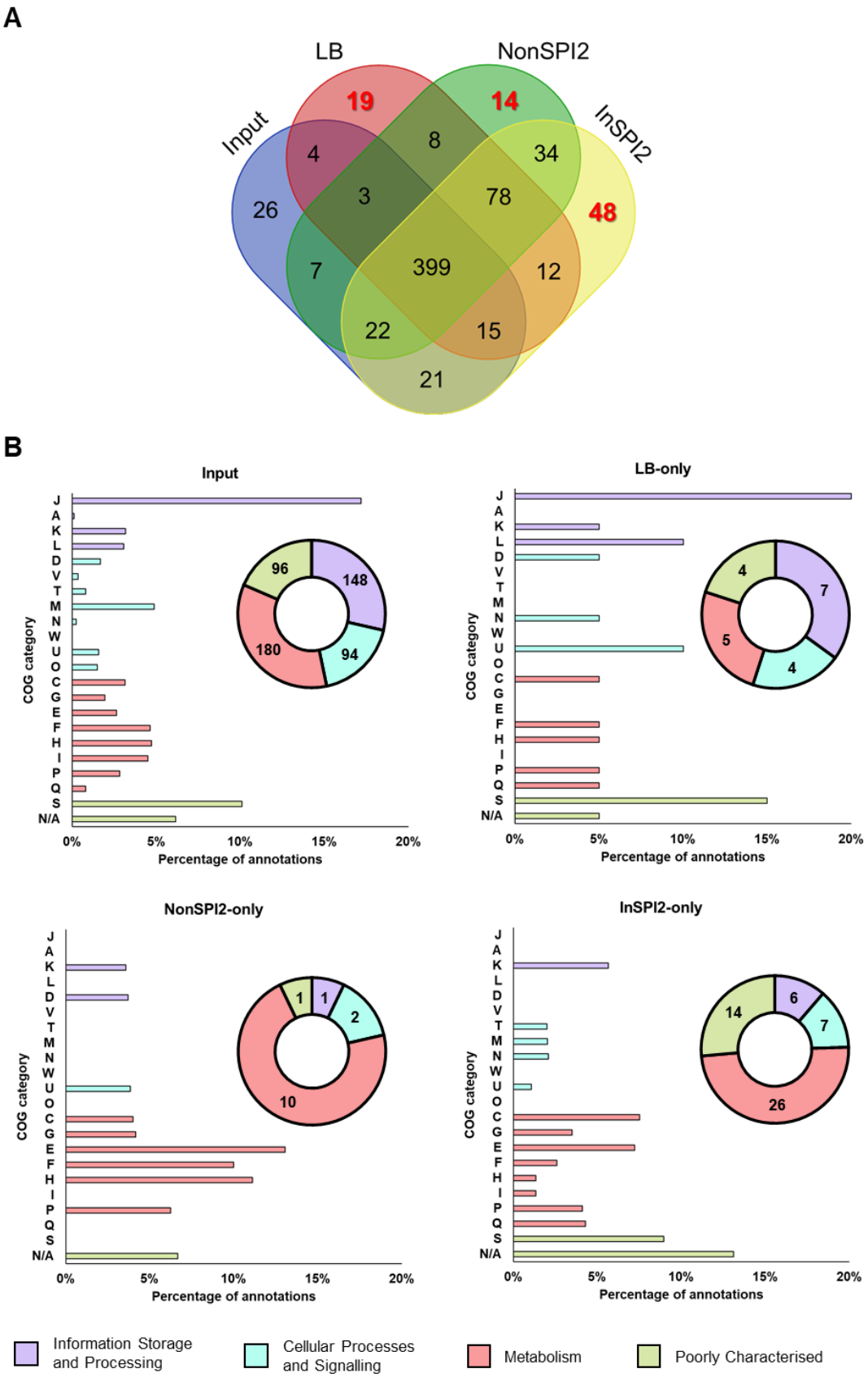

Figure 4.3 Genetic requirements of *S.* Enteritidis P125109 for optimal growth in LB, NonSPI2 and InSPI2

A. Venn diagram comparing the P125109 required genes in LB, NonSPI2 and InSPI2 growth conditions. Numbers in red indicate genes that were only required in the specific growth media. Gene lists are available in **Appendix 4**. **B.** Changes in proportion of required genes assigned to each COG category. Doughnut charts (insets) show the distribution of COG annotations in the four major categories, with total numerical counts in each major category shown. Input bar chart shows COG annotations from all 497 genes identified as required in the input, whereas the other three bar charts only considered the genes specific to that growth media. Definitions of COG categories are given in **Table 4.3**. Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/. N/A, not assigned.

In **Figure 4.3B**, the distribution of LB-only required genes into the four major categories ("Information Storage and Processing", "Cellular Processes and Signalling", "Metabolism" and "Poorly Characterised") is similar to that of the input distribution, although certain COG categories were not represented (e.g. RNA processing and modification, A; defense mechanisms, V). Compared to the NonSPI2-only and InSPI2-only fewer metabolism-related genes were required for optimal growth in LB, a reflection of the nutrient-rich LB environment (Sezonov *et al.*, 2007). Among the 19 LB-only required genes, 6 genes (*xseB*, *cydA*, *recB*, *gidA*, *yjeA*, *arcA*) have been previously identified as required by *S*. Typhi for growth in LB (Langridge *et al.*, 2009). The *rec* gene, encoding an exonuclease subunit, was also reported to be required by *S.* Typhimurium 14028 (Khatiwara *et al.*, 2012) and different *E. coli* isolates (Rousset *et al.*, 2021) after several passages in LB. The identification of *rec* gene in multiple TIS screens highlights the importance of this gene for fitness in the LB environment.

The genes that were required for optimal growth in NonSPI2 and/or InSPI2 were prominently dominated by metabolism-related genes, confirming the nutritional deficiencies encountered by the bacteria grown in a synthetic minimal media. Genes required for optimal growth in both NonSPI2 and InSPI2 included *pur* genes (for purine biosynthesis) and *aro* genes (for aromatic amino acids biosynthesis). In InSPI2-only required genes, several genes associated with SPIs and RODs were

required; the *dksA* gene was also identified, which has been experimentally validated as required for growth of *S.* Typhimurium in minimal medium (Azriel *et al.*, 2015).

4.2.3 Genetic requirements of D7795

4.2.3.1 Identification of genes required for *in vitro* growth

Essentiality analysis of the D7795 input library identified 467 required genes, 3776 dispensable genes, 238 ambiguous genes and 720 short genes (**Figure 4.4A**). Of the 467 required genes (**Appendix 3**), 465 genes were encoded in the chromosome and 2 genes (*samA* and *SEN_p0046*) were located in the virulence plasmid pSEN-BT; no required genes were identified on the smaller plasmid pRGI00316.

COG categories were assigned to the D7795 reference genome as described earlier and in Section 2.8.2. Similar to the observations in P125109, the majority of the D7795 required genes are involved in translation (J, 18%), cell wall biogenesis (M, 9%), and nucleotide metabolism (F, 7%). Nine required genes were associated with pathogenicity islands, with four SPI-2 genes (*ssaT*, *ssaH*, *ttrAB*), two SPI-6 genes (*SEN2077* and *SEN2078*), two SPI-10 genes (*SEN4248* and *SEN4250*), and one SPI-14 gene (*SEN0801*) identified.

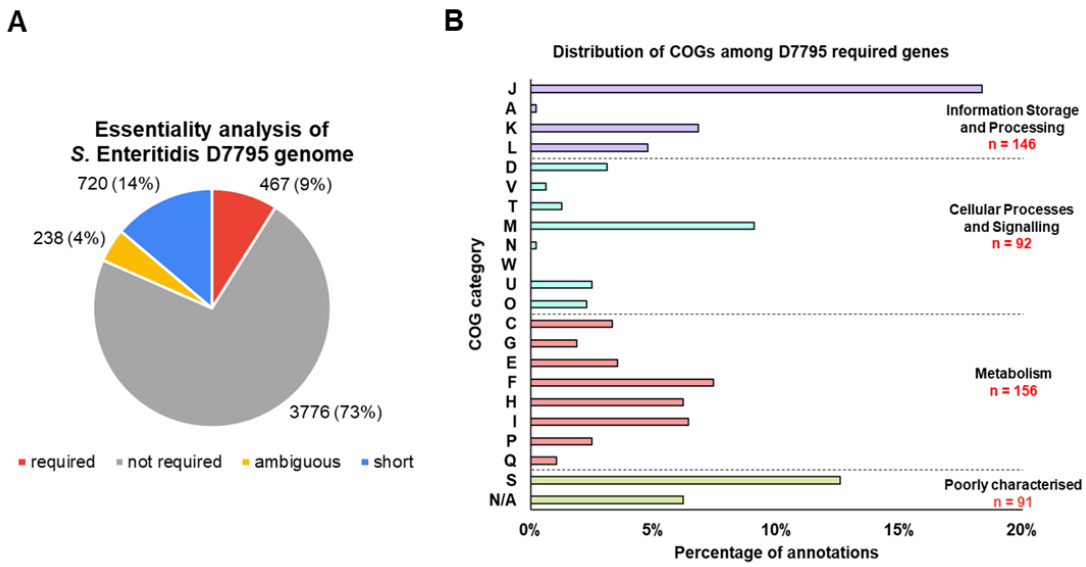

Figure 4.4 Essentiality analysis of *S.* Enteritidis D7795 genome and distribution of functional categories

A. Pie chart showing distribution of required, dispensable (not required), ambiguous and short categories of genes. **B.** Bar chart showing the distribution of the 467 D7795 required genes across the COG categories, as percentage of each category out of the total annotations assigned to required genes. The numbers in red indicate the total numerical counts of COG annotations in each category. Definitions of the COG categories are given in **Table 4.3**. N/A, not assigned.

4.2.3.2 Genetic requirements for optimal growth in LB, NonSPI2 and InSPI2 media

D7795 genes required for optimal growth in LB, NonSPI2 and InSPI2 were identified as discussed in Section 4.2.2.2. Following two passages in the respective growth media, 541 genes, 559 genes and 532 genes were designated as required in LB, NonSPI2 and InSPI2 respectively. After removing genes that had been identified as required in the input , there are 23 LB-only required genes, 77 NonSPI2-only required genes and 47 InSPI2-only required genes (**Figure 4.5A, Appendix 5**).

Five genes identified as required in D7795 for LB growth were also reported by Barquist *et al.* (2013) (*ppiB*, a peptidyl-prolyl isomerase; *cydA*, cytochrome oxidase d subunit and *crp*, cAMP-activated global transcriptional regulator) and Canals *et al.* (2019) (*rstB*, the sensor kinase of the *rstAB* two-component system, and *rnfD*, a component of the electron transport chain).

Most of the genes identified as required for optimal growth in NonSPI2 and InSPI2 minimal media were, as expected, metabolism-related, with genes involved in amino acid metabolism (E) and nucleotide metabolism (F) and energy production (C) predominantly identified. Similar to P125109, the D7795 *dksA* gene was also identified as required for growth in InSPI2 media. Several SPI-1 and SPI-2 genes and a SPI-1 effector SopE were found to be required for growth in NonSPI2, while two SPI-5 genes (*pipB* and *sORF26*) and one ROD9 genes (*SEN1001*) were required for growth in InSPI2.

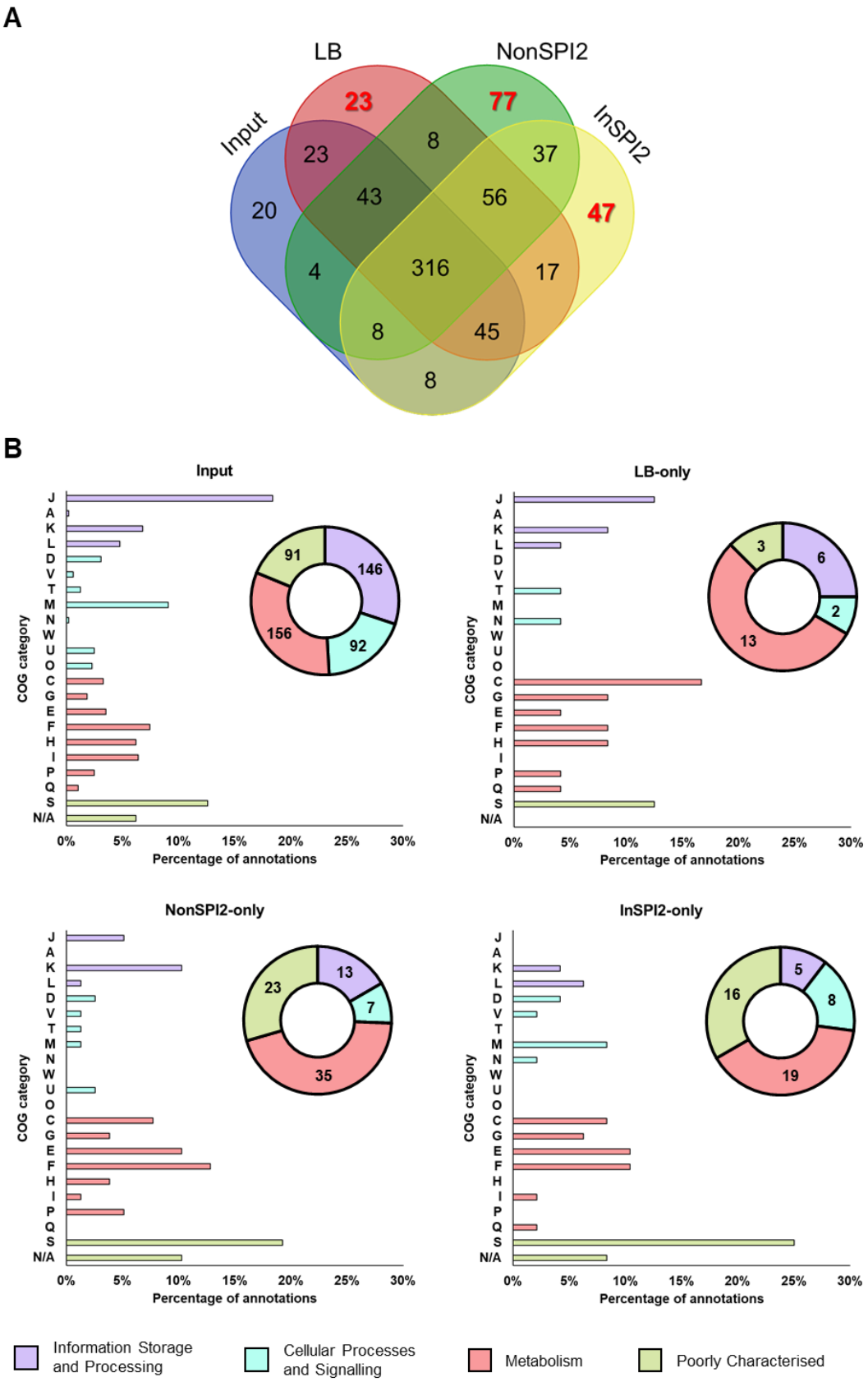

Figure 4.5 Genetic requirements of *S.* Enteritidis D7795 for optimal growth in LB, NonSPI2 and InSPI2

A. Venn diagram comparing the D7795 required genes in LB, NonSPI2 and InSPI2 growth conditions. Numbers in red indicate genes that were only required in the specific growth media. Gene lists are available in **Appendix 5**. **B.** Changes in proportion of required genes assigned to each COG category. Doughnut charts (insets) show the distribution of COG annotations in the four major categories, with total numerical counts in each major category shown. Input bar chart shows COG annotations from all 467 genes identified as required in the input, whereas the other three bar charts only considered the genes specific to that growth media. Definitions of COG categories are given in **Table 4.3**. Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/. N/A, not assigned.

4.2.4 Cross-species comparisons of genes required for growth

4.2.4.1 Common required genes shared between *S.* Enteritidis, other *Salmonella* serovars and *E. coli*

To put our essentiality analysis in context, the *S.* Enteritidis genes required for growth *in vitro* from the input libraries (Sections 4.2.2.1 and 4.2.3.1) was compared with genetic requirements identified in other *Salmonella* serovars, and the closely related *E. coli*. Two required gene sets were chosen: the first is a list of 228 essential genes shared between *S*. Typhimurium, *S*. Typhi and *E. coli* (Barquist *et al.*, 2013b), with the *Salmonella* genes identified by TraDIS and *E. coli* genes experimentally validated by their absence from the KEIO collection, a library of single-gene knockout mutants (Baba *et al.*, 2006). The second list of required genes was generated by a study on the essential genome of *E. coli* K-12 (Goodall *et al.*, 2018), which reported 248 genes identified in common by three separate sources: TraDIS, KEIO collection and the Profiling of *E. coli* Chromosome (PEC) database, a compilation of available literature on the *E. coli* essential genome (Yamazaki *et al.*, 2007).

Figure 4.6 shows the comparison of the P125109 or D7795 *in vitro* required genes with the required gene sets from Barquist *et al* (2013) and Goodall *et al.* (2018). Each *S.* Enteritidis gene list showed significant overlap with the published datasets, validating our TIS approach. The 207 genes shared between *S.* Enteritidis P125109, *S.* Enteritidis D7795, *S.* Typhimurium, *S.* Typhi and *E. coli* K-12 are concerned with vital cellular processes, and likely represent true essential genes (examples in **Table**

4.4; full list available in **Appendix 6**). I note that five “short” genes (gene length less than 200 nt) in either *S.* Enteritidis strain were identified as required by both Barquist *et al.* (2013) and Goodall *et al.* (2018), namely *infA*, *csrA*, *rpmD*, *rpmC* and *rpmH*.

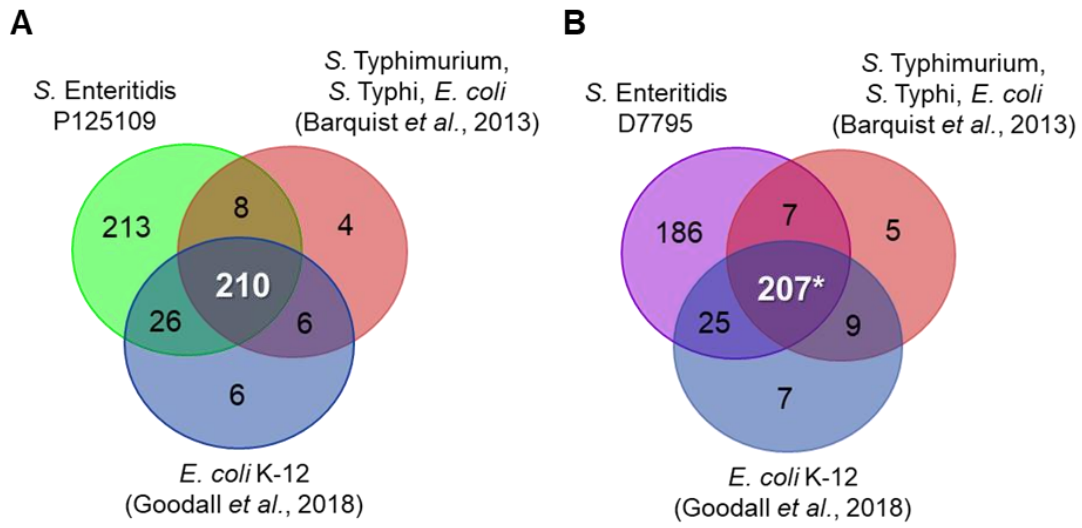

Figure 4.6 Comparison of required genes identified in *S.* Enteritidis P125109 and D7795 with published gene essentiality studies in other *Salmonella* serovars and *E. coli*

Only P125109 and D7795 genes with orthologues in S. Typhimurium LT2 were used in the comparison (**Appendix 6**). The asterisk (*) indicates that all the 207 D7795 genes were included in the 210 required P125109 genes.

Table 4.4 Examples of the 207 required genes that were required in *S.* Enteritidis P125109 and D7795 and reported in published gene lists (Barquist *et al.*, 2013; Goodall *et al.*, 2018)

COG category and description		Function	Genes
J	Translation, ribosomal structure and biogenesis	Ribosomal proteins	*rplBCDEFJLMNOPQRSTUVWX* *rpmAB* *rpsABCDEGHIJK**L**MNPQ**S***
		rRNA processing	***yqgF***
		Aminoacyl tRNA-synthetases	*argS*, *asnS*, *aspS*, *cysS*, *glnS*, *hisS*, *leuS*, *pheS*, *pheT*, *proS*, *serS*, *thrS*, *tyrS*, *valS*
		Initiation, elongation, and peptide chain release factors	*infBC*
K	Transcription	RNA polymerase	*rpoABCH*
		Transcriptional regulators	*lexA*
L	Replication, recombination and repair	Primosome associated	*dnaAC*, *ssb*
		DNA polymerase III	*dnaEN*, *holAB*
		Gyrase and topoisomerase	*gyrAB*, *parE*
D	Cell cycle control, cell division, chromosome partitioning	Cell division proteins	*ftsALQWZ* *zipA*
M	Cell wall / membrane / envelope biogenesis	Peptidoglycan synthesis	*murABCDEFG*, *mraY*
		Lipoprotein transport	*lolAB*
C	Energy production and conversion	Electron transport chain	*rnfBCD*
G	Carbohydrate transport and metabolism	Glycolysis	*fba*
E	Amino acid transport and metabolism	Amino acid biosynthesis	*dapABDE*
F	Nucleotide transport and metabolism	Purine metabolism	*adk*, *gmk*
		Pyrimidine metabolism	*dut, tmk*
H	Coenzyme transport and metabolism	Protoporphyrin-IX biosynthesis	*hemABCG*
		Riboflavin biosynthesis	*ribDEFH*

The gene highlighted in **bold** was only identified in P125109, and not D7795.

4.2.4.2 Differences in requirements for orthologous genes in *S.* Enteritidis and *S.* Typhimurium

The comparison method used in Section 4.2.4.1 is a common and logical approach for the identification of required orthologous genes shared between *Salmonella* serovars and related bacterial species. However, the differences that arise from such comparisons can be challenging to interpret, in part due to the differences in experimental protocols, transposon library profiles and bioinformatic processing pipelines used by different laboratories.

The Hinton Laboratory recently published a TIS-based analysis of the genetic requirements of African *S.* Typhimurium D23580 (introduced in Section 1.5) for survival and growth both *in vitro* and during macrophage infection (Canals *et al.*, 2019a). The experiments performed in this chapter have closely followed the experimental design described in Canals *et al.* (2019a), making the Canals *et al.* (2019a) dataset the ideal direct comparator for the two *S.* Enteritidis datasets generated in this investigation. This three-strain comparison between *S.* Enteritidis P125109, D7795 and *S.* Typhimurium D23580 was conducted to identify any required genes that are specific to the *S.* Enteritidis serovar, or the two African strains associated with invasive disease. However, a simple overlaying of the common genes between the three strains by means of Venn diagrams was complicated by the vastly different insertion densities of the two *S.* Enteritidis transposon libraries (~200,000 each) and the D23580 library (at least ~500,000).

To address the issue of different transposon library densities, a customised pipeline (described in Section 2.8.4.5) was used to compare the three strains and to perform essentiality analysis. The TIS libraries used for the inter-strain essentiality analysis included libraries generated in experiments described in Chapter 5, namely (LB) input, LB output (representing mutants recovered after a 10-h growth in LB) and

macrophage output (representing mutants recovered after 12-h infection in macrophages). The NonSPI2 and InSPI2 output libraries were omitted from this customised inter-strain essentiality analysis because of the substantial decrease in the number of unique transposon insertions as compared to the input, particularly for the D7795 samples (**Table 4.2**).

Assuming that genes which are required would appear as required regardless of the experimental condition, inter-strain essentiality analysis was performed using both essentiality calls and changes in abundance of read counts for all three conditions (input, LB output, macrophage output).

Sixty-three genes were identified that were not required in all three strains. These genes were termed “differentially required”, and can be broadly grouped into five clusters (**Figure 4.7**). All of the genes identified have lengths longer than 200 nt, indicating that their essentiality calls should be reliable (see Section 2.8.4.3). Identification of *cysS* as not required in *S.* Typhimurium D23580 supported the inter-strain essentiality analysis approach, as Canals *et al.* (2019a) showed experimentally that CysS is dispensable for D23580.

The clusters of particular interest are clusters 3, 4 and cluster 5. Cluster 3 represents genes that are not required by the two African *Salmonella* strains D7795 and D23580 but are required in the Global Epidemic strain P125109. Cluster 4 shows genes that are required in both *S.* Enteritidis strains but not in Typhimurium D23580, and includes *cysS* as mentioned above. Cluster 5 involves genes that are dispensable in *S.* Enteritidis but required in *S.* Typhimurium D23580, and includes genes belonging to the iron-sulphur cluster (ISC) system (*yfhP* [*iscR*], *hscA* and *fdx*) and several SPI-2 genes (*SEN1635* and *ssaR*). Further investigation of the respective clusters of genes should reveal more insights into the differential patterns of genes required.

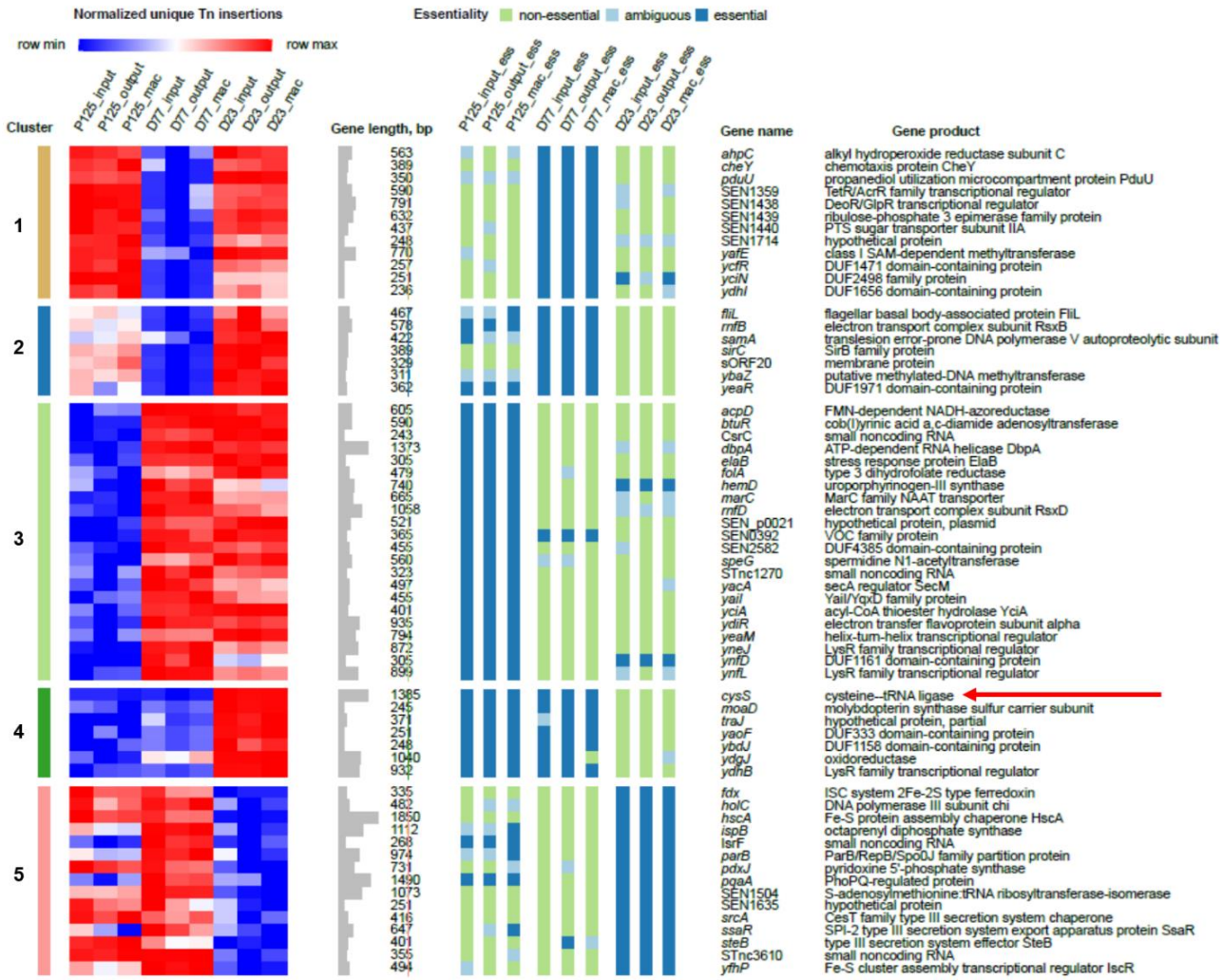

Figure 4.7 Identification of differentially required genes between *S.* Enteritidis P125109, *S.* Enteritidis D7795 and *S.* Typhimurium D23580

Inter-strain essentiality analysis was performed as described in 2.8.4.5, using transposon insertion read counts (represented as heat map on the left side, with red indicating many insertions and blue indicating very few insertions) and essentiality calls from LB input, LB output and macrophage output libraries (represented by green and blue squares). The names of the individual libraries are indicated above each coloured column. The *cysS* gene is highlighted with a red arrow. Figure was generated by Dr Alex Predeus.

4.2.4.3 Differences in *S.* Enteritidis gene requirements for optimal growth in LB, NonSPI2 and InSPI2

Comparison of the genes required for optimal growth in LB, NonSPI2 or InSPI2 only between *S.* Enteritidis P125109 and D7795 revealed few overlaps in gene requirements (**Figure 4.8**). Although the differences between insertion densities of the output libraries likely exaggerated the differences in gene requirements, there was also experimental evidence to support that D7795 responds to nutritional limitations differently from P125109.

In growth curve experiments to determine the ESP time point for passaging the transposon libraries, D7795 was observed to grow much more slowly than P125109 in both NonSPI2 and InSPI2 media (**Figure 4.9**). Whereas slow(er) growth in InSPI2 media is expected (due to the combination of low phosphate and acidic pH), bacterial growth in NonSPI2 media (with 25 mM phosphate and at pH 7.4) should be relatively fast. Indeed, growth measurements of *S.* Typhimurium in NonSPI2 and InSPI2 media indicated that bacterial growth in NonSPI2 is faster than growth in InSPI2 conditions; however, the InSPI-2 grown culture still reached a maximum OD_{600} of around 1.0 at approximately 8 h after inoculation (Löber *et al.*, 2006). In contrast, wild-type D7795 grown in InSPI2 media reached a maximum OD_{600} below 0.60 at around 20 h after inoculation.

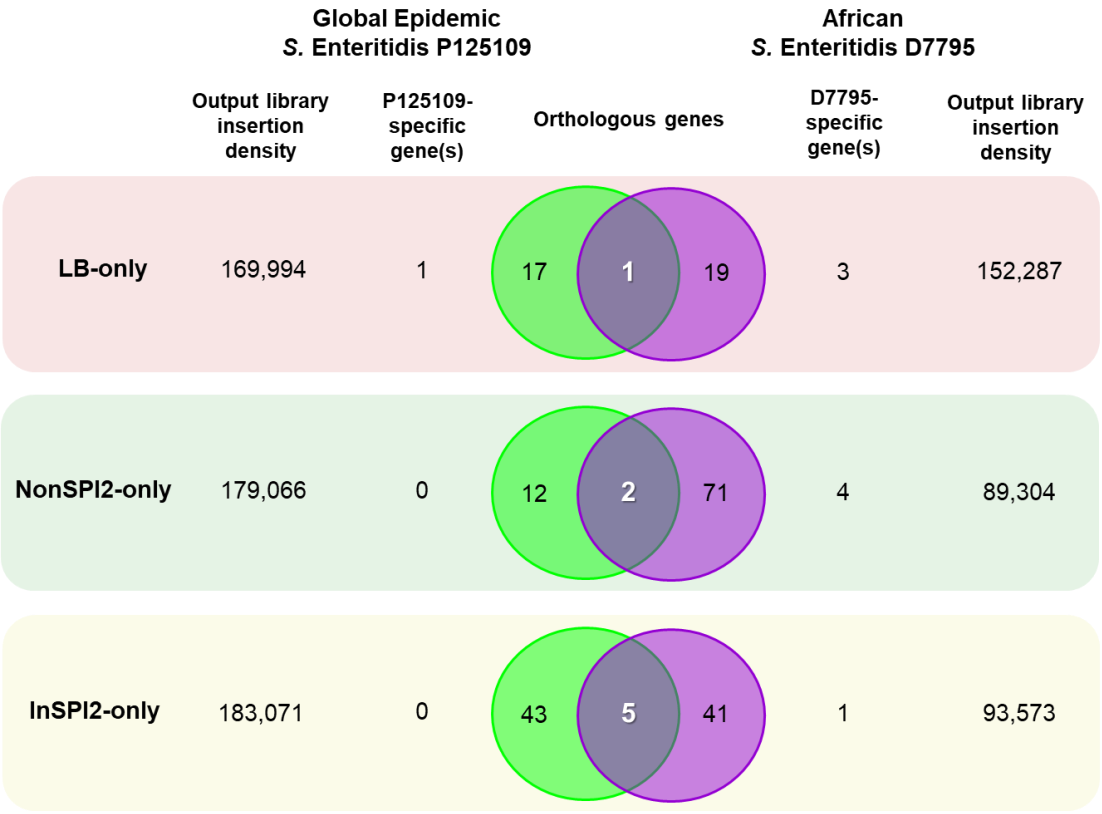

Figure 4.8 Comparison between *S.* Enteritidis P125109 and D7795 gene requirements for optimal growth in LB, NonSPI2 and InSPI2 media

LB-only, NonSPI2-only and InSPI2-only genes in each strain have been previously defined in Sections 4.2.2.2 and 4.2.3.2. P125109-specific and D7795-specifc genes refer to genes that have no orthologues in the other strain. Gene lists are available in **Appendix 7**.

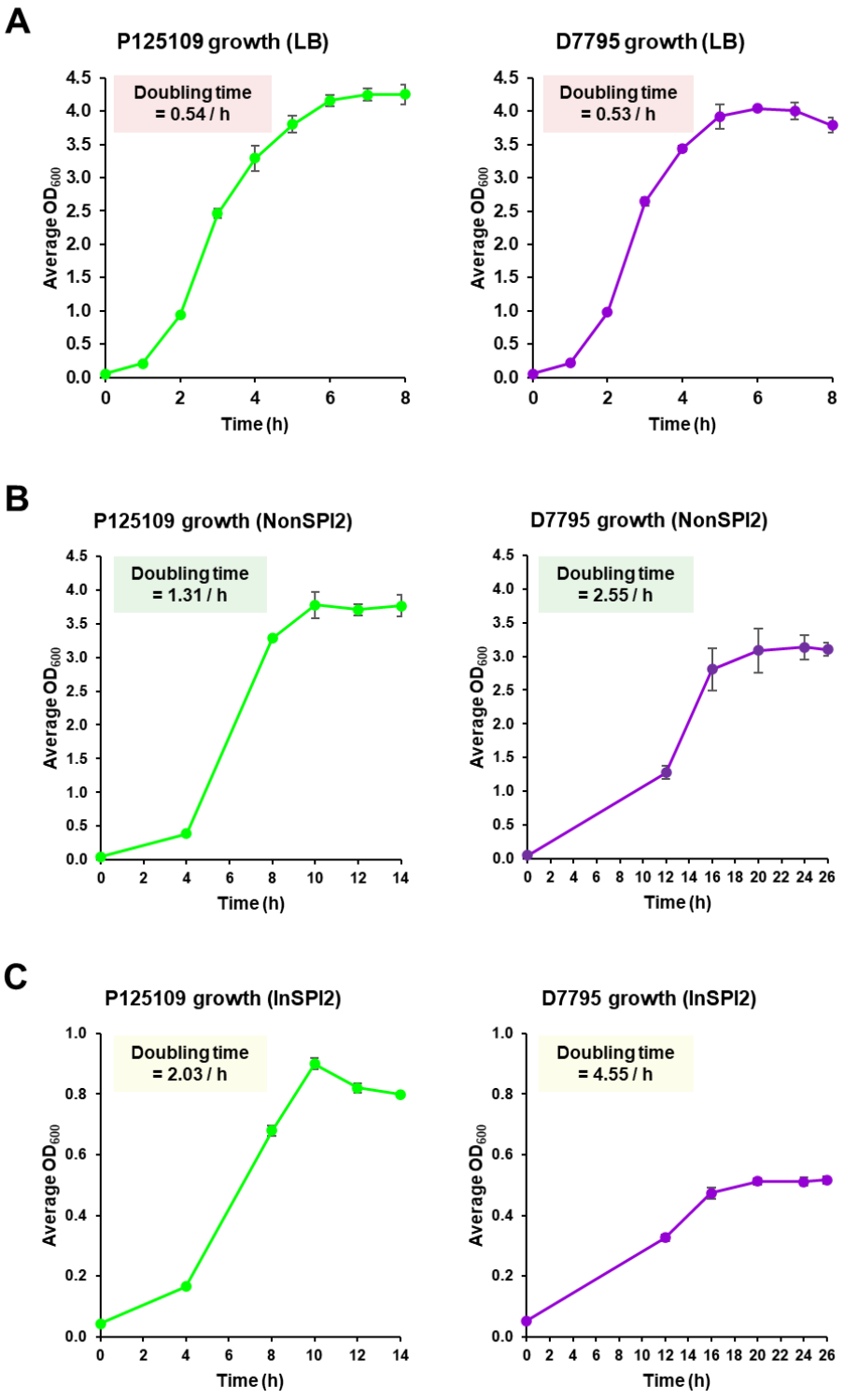

Figure 4.9 Growth curves of wild-type *S.* Enteritidis P125109 and D7795 in LB, NonSPI2 and InSPI2 media

Growth curves of wild-type P125109 and D7795 were performed in LB, NonSPI2 and InSPI2 media (as described in Section 2.3.2) to establish the early stationary phase (ESP) timepoints for the *in vitro* passage experiments. Graphs show the average values from three biological replicates, with error bars showing standard deviation. Doubling time was determined from OD_{600} measurements at the beginning and end of exponential growth (estimated by plotting ln [OD_{600}] against time). Growth rates (*r*) were calculated by the formula $r = (\ln[OD2/OD1])/(T2-T1)$ and the doubling time corresponds to $\ln(2)/r$.

4.2.4.4 Strain-specific genes required for growth

Finally, many of the required genes present in only P125109 or D7795 were prophage genes (**Appendix 1**), although none of the genes appear to encode prophage repressors. Prophage repressors are commonly found to be required genes for growth in TIS studies because inactivation leads to prophage de-repression and phage-mediated cell lysis (Barquist *et al.*, 2013b; Canals *et al.*, 2019a). Interestingly, several genes from the ROD21 region (introduced in Section 1.4.1.6), which is absent in D7795, were required for *in vitro* growth in P125109.

4.3 Discussion

The use of the high-throughput TIS technique allowed us to survey the genetic requirements of *S.* Enteritidis strains P125109 and D7795 during growth in three different conditions. To our knowledge, this is the first comparative study investigating *in vitro* required genes of P125109 and D7795.

The required genes identified from analysis of the input libraries were largely genes necessary for *Salmonella* survival and growth, and the corresponding transposon insertions were absent from the initial library (prior to any experimental selection). A key finding from this chapter was the identification of a common set of 207 required genes shared between P125109 and D7795 and other *Salmonella* serovars (**Figure 4.6** and **Table 4.4**), revealing that the two *S.* Enteritidis strains share a core gene set for survival. These 207 required genes mainly encode the basic cellular machinery (e.g. DNA replication and protein translation) and pathways vital for the growth of the bacteria, including cell wall biogenesis and cell division. Similar conservation of required genes among bacterial strains within the same species or between different species and phyla has been reported (Barquist *et al.*, 2013b; Canals *et al.*, 2012; Grazziotin *et al.*, 2015; Rousset *et al.*, 2021).

Gene requirements can also be influenced by various factors such as environmental condition or genetic background (Rancati *et al.*, 2018). By comparing the required genes in *S.* Enteritidis P125109 and D7795 with those identified in African *S.* Typhimurium strain D23580 (Canals *et al.*, 2019a), we identified 63 genes that were differentially required across the three strains (**Figure 4.7**). The 22 genes identified as dispensable in the two African *Salmonella* strains (cluster 3) are of particular importance. Pseudogenisation and the associated loss of gene function has been linked to a restriction in host range of *Salmonella* (as observed in *S.* Typhi) (Sabbagh *et al.*, 2010) and/or a move away from an enteric lifestyle to being extra-intestinal (Okoro *et al.*, 2015; Pulford et al., 2020). In the future, determining whether the functions of these 22 genes are truly dispensable for *S*. Enteritidis D7795 and *S.* Typhimurium D23580 would be important.

Several genes in the SPI regions were identified as required for *in vitro* growth in either *S.* Enteritidis strain (Sections 4.2.2.1 and 4.2.3.1). While previous TIS studies in *S.* Typhimurium have also reported a requirement of some SPI genes for growth in laboratory conditions (Barquist *et al.*, 2013b; Canals *et al.*, 2019a), the generation of viable null *S.* Typhimurium and *S.* Enteritidis mutants in various genes located at SPI regions suggest that these genes are not truly required genes in both *Salmonella* serovars (Barquist *et al.*, 2013b; Canals *et al.*, 2019a; Elsheimer-Matulova *et al.*, 2015; Hensel *et al.*, 1998; Porwollik *et al.*, 2014; Rychlik *et al.*, 2009; Santiviago *et al.*, 2009). A possible explanation for the fewer transposon insertions in the “required” SPI genes is the binding of the histone-like nucleoid structuring (H-NS) protein, encoded by the *hns* gene, to the SPIs thereby preventing transposon access (Kimura *et al.*, 2016).

Horizontally-acquired genes such as SPIs are characterised by their lower GC content (Section 1.4.1), and in many bacterial species, H-NS preferentially binds such

AT-rich regions in the genome as a mechanism to silence the expression of foreign genes (Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Will *et al.*, 2015). H-NS binding to SPI regions has been previously reported in *S.* Typhimurium (Lucchini *et al.*, 2006; Navarre *et al.*, 2006). A comparison of the binding pattern of H-NS detected in studies using *S.* Typhimurium (Dillon *et al.*, 2010; Lucchini *et al.*, 2006; Navarre *et al.*, 2006) with the *S.* Enteritidis required genes identified in this chapter should help to clarify if there is a correlation between H-NS binding and low number or absence of transposon insertion in the respective *S.* Enteritidis genomes.

Following passages of the transposon libraries in LB, NonSPI2 and InSPI2 media, additional genes required for the survival and growth of the respective *S.* Enteritidis strains were identified (**Figure 4.3** and **Figure 4.5**). A key observation from these findings is that the gene requirements for growth in the respective growth media differ greatly between P125109 and D7795 (**Figure 4.8**), albeit likely to be over-estimated due to the lower insertion densities of the NonSPI2 and InSPI2 output libraries, which in turn affect the robustness of essentiality calls (Chao *et al.*, 2016). Indeed, Feasey *et al.* (2016) has reported that carbon utilisation patterns of D7795 and P125109 differ, observations that have been corroborated by experiments performed by fellow members of the Hinton Laboratory (Bowers-Barnard, 2018). Whether the observed gene differences reflect the different metabolic pathways used by P125109 and D7795 will be an important area for future investigation.

As with all global mutagenesis approaches, the strategy presented in this chapter has certain limitations. The relatively low density of the transposon libraries meant that approximately 14% of each *S.* Enteritidis genome could not be reliably assigned an essentiality call. Although the list of “short” genes only contained five required genes identified in other gene essentiality studies (Section 4.2.4.1), it is possible that more of the approximately 700 “short” genes will prove to be required In the future.

Overall, exposure of the *S.* Enteritidis transposon libraries to three *in vitro* growth conditions has successfully identified genes that were conditionally required or differentially required by the two *S.* Enteritidis strains for survival and growth in particular environmental conditions. The *S.* Enteritidis transposon libraries represent a valuable resource that can be used to study gene function during the process of infection itself, as will be investigated in the next chapter.

Chapter 5 :

Fitness landscape of African and Global Epidemic *Salmonella* Enteritidis during infection of RAW 264.7 macrophages

5.1 Introduction

Survival and replication within macrophages is a hallmark of *Salmonella* pathogenicity and central to the establishment of systemic infection (Fields *et al.*, 1986; Petersen and Miller, 2019). Consequently, macrophage infection offers an approach for studying *Salmonella* virulence that permits the avoidance of whole animal experiments (Tsolis *et al.*, 2011).

To understand how African *S*. Enteritidis D7795 causes disease, it is important to determine whether this pathovariant carries novel virulence genes that have not been described previously in Global Epidemic isolate P125109 or other *Salmonella* serovars. To date, two genome-wide studies involving *S.* Enteritidis P125109 have defined the genes required for infection of mice (Silva *et al.*, 2012), human epithelial cells, chicken liver cells and chicken macrophages (Shah *et al.*, 2012). In the case of African *S.* Enteritidis D7795, Feasey *et al.* (2016) remains the only study to examine the virulence of D7795 in an infection model. In chickens, D7795 was found to be less invasive and had lower levels of systemic dissemination to the liver and spleen, than a gastroenteritis-associated *S.* Enteritidis isolate, while displaying similar levels of intestinal colonisation (Feasey *et al.*, 2016). A comprehensive description of genes required for macrophage infection, the primary intracellular niche during systemic infection, is still lacking.

In this chapter, the P125109 and D7795 transposon libraries were screened in RAW 264.7 murine macrophages to determine the genes required for macrophage infection (**Figure 5.1**). The findings were then compared with the genetic requirements of African *S.* Typhimurium D23580 for macrophage infection, reported in Canals *et al.* (2019a), and three other genome-wide analyses of *S.* Typhimurium and *S.* Enteritidis in murine infection, to determine if there are novel virulence genes that had not been previously described. As previously mentioned, none of the virulence phenotypes

reported in this chapter were validated experimentally, due to the disruption to research activities as a result of the COVID-19 pandemic-related lockdown implemented in March 2020.

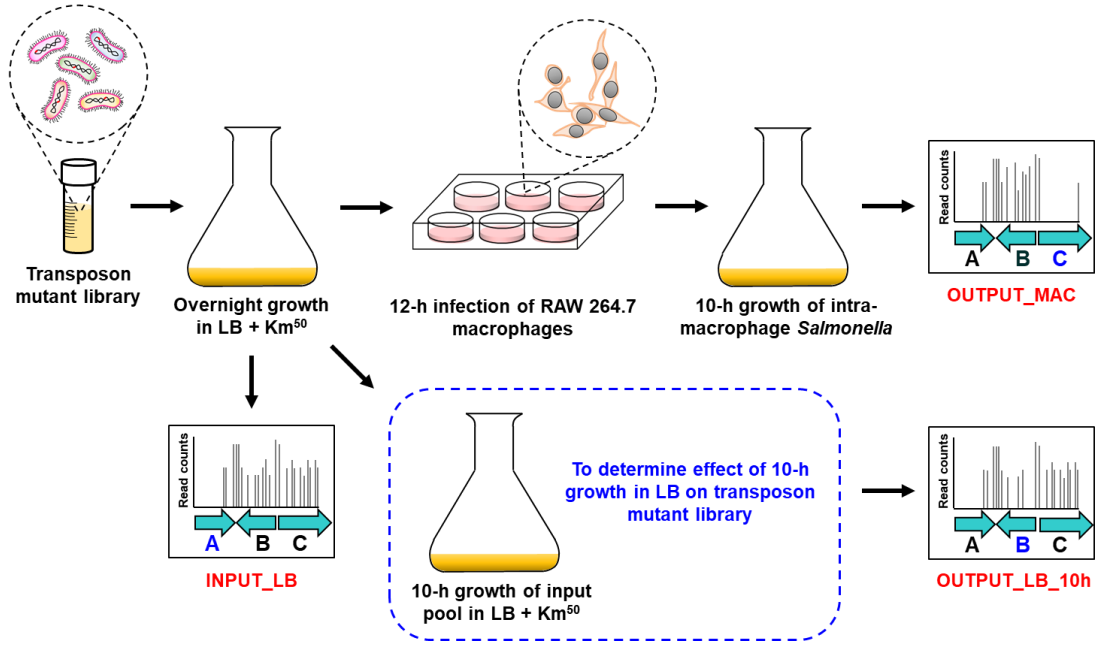

Figure 5.1 Schematic representation of the assay of *S.* Enteritidis transposon libraries in RAW 264.7 macrophage infection

Experimental details are provided in Section 2.7.3. Genomic DNA from the input and output samples was purified and sequenced by Illumina technology. The sequence reads were mapped to the P125109 or D7795 reference genome to identify unique transposon insertion sites. The genes (*A*, *B*, or *C*) highlighted in colour illustrate genes that are required for *in vitro* growth (gene *A*), or contribute to fitness in LB growth (gene *B*) or in macrophage infection (gene *C*).

5.2 Results

5.2.1 Intra-macrophage survival and replication of *S.* Enteritidis P125109 and D7795

To investigate the virulence phenotypes of *S.* Enteritidis P125109 and D7795 during RAW 264.7 macrophage infection, the levels of intra-macrophage replication of the respective *S.* Enteritidis wild-type strains was determined with the gentamicin-protection assay. Two additional strains, A1636 and CP255, were included in this experiment: A1636 is a Global Epidemic clade isolate from Africa, while CP255 belongs to the same Central/Eastern African clade as D7795 (Feasey *et al.*, 2016; Perez-Sepulveda *et al.*, 2021).

The PhoPQ two-component regulatory system is required for the survival of *S.* Typhimurium within macrophages (Petersen and Miller, 2019; Thompson *et al.*, 2011), and a genome-wide screen of P125109 Tn*5* mutants showed that Δ*phoPQ* mutants were negatively selected during murine infection (Silva *et al.*, 2012). To generate negative controls for the intra-macrophage replication assays, I constructed Δ*phoPQ* mutants of strains P125109 and D7795 (Section 2.4.5). *S.* Typhimurium strains 4/74 and 4/74 Δ*phoPQ* were included as additional positive and negative controls to confirm that the experiment had performed as expected.

The gentamicin-protection assays showed that all the *S.* Enteritidis and *S.* Typhimurium strains were taken up by RAW 264.7 macrophages at similar levels. At 15 h post-infection, the Central/Eastern African isolates displayed significantly higher levels of intra-macrophage replication than Global Epidemic clade isolates (**Figure 5.2**). To my knowledge, this is the first report that Central/Eastern African clade strains are more virulent than Global Epidemic clade during infection of mammalian cells.

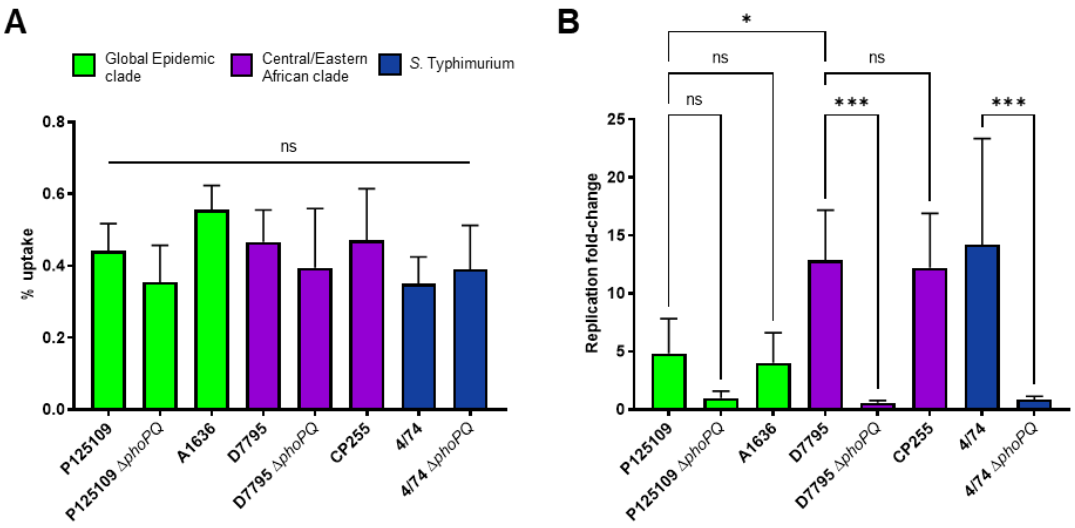

Figure 5.2 Central/Eastern African *S.* Enteritidis strains replicate faster than Global Epidemic clade strains during RAW 264.7 macrophage infection

Intra-macrophage replication assays were performed as described in Section 2.5.2. *S.* Enteritidis strains are colour-coded by clades, according to Feasey *et al.* (2016): green, Global Epidemic; purple, Central/Eastern African. The *S.* Typhimurium 4/74 and 4/74 Δ*phoPQ* (blue) strains were included as additional controls. **A.** Uptake of *Salmonella* strains by RAW 264.7 macrophages, shown as the percentage of the infecting inoculum recovered (CFU) at 1.5 h post-infection (p.i.). **B.** Intra-macrophage replication of *Salmonella*, shown as the ratio of intracellular bacteria (CFU) recovered at 15.5 h p.i. compared to the CFU recovered at 1.5 h p.i., as fold-change. Both panels **A** and **B** represent average values obtained from five independent experiments with three replicates each, and error bars show standard deviation. Statistical tests used were one-way ANOVA, followed by Bonferroni's multiple comparison test to compare selected pairs of means. ns, $P \geq 0.05$; *, $P < 0.05$; ***, $P < 0.001$. The lack of statistical significance of the difference between the values for P125109 wild-type and Δ*phoPQ* mutant may reflect the large variation in CFU counts that were obtained between the independent biological replicates.

5.2.2 Assaying P125109 and D7795 transposon libraries in macrophage infection

The P125109 and D7795 transposon libraries were used to investigate the process of intracellular infection of RAW 264.7 macrophages. Each pool of transposon mutants was grown in LB (input) then passaged once through murine macrophages. Multiple passages were not done because a similar screen of a *S.* Typhimurium strain D23580 transposon library showed that additional passages selected for LPS-defective mutants (Canals *et al.*, 2019a). Intra-macrophage bacteria were recovered at 12 h post-infection. and grown in LB for 10 h to generate the output sample (output_MAC).

Chronologically, the TIS screen of the *S.* Enteritidis transposon libraries in macrophage infection was completed before the investigation of genetic requirements for growth *in vitro* (Chapter 4), meaning that knowledge of which genes were required by the respective *S.* Enteritidis strains for growth in LB were not yet known. Consequently, a fraction of the input was sub-cultured in LB for 10 h to ascertain the effect of growth in LB broth culture on the composition of the transposon library (output_LB_10h).

Genomic DNA from the input and output samples was purified and prepared for Illumina sequencing of DNA adjacent to the transposon, as described in Section 2.7.4. A total of 12 DNA libraries were sequenced on a single lane of an Illumina HiSeq4000, generating a total of 340,375,208 paired-end reads. **Table 5.1** shows the number of sequence reads that contained the transposon tag sequence, and the sequence reads that were uniquely mapped to the respective *S.* Enteritidis genomes. The complete data sets are available for visualisation in JBrowse genome browsers at the following URLs: **hintonlab.com/jbrowse/index.html?data=tradis_P125109/data** and **hintonlab.com/jbrowse/index.html?data=tradis_D7795/data**.

Table 5.1 Transposon insertion sequencing (TIS) data set derived from *S.* Enteritidis P125109 and D7795 transposon libraries used for macrophage infection experiment

Sample	Demultiplexed reads with transposon (Tn) tag	Reads aligned to *Salmonella* genome (%)	Deduplicated reads	Deduplicated reads with *Salmonella* sequence next to Tn tag	Reads mapped to annotated features (%)	Unique insertions[a]	Unique insertions in annotated features (%)
P125109_input_LB_1	8,575,727	8,324,993 **(97.1)**	4,534,535	4,196,203	3,804,340 **(90.7)**	189,401	152,454 **(80.5)**
P125109_input_LB_2	5,617,456	5,385,580 **(95.9)**	2,491,049	2,228,727	2,038,401 **(91.5)**		
P125109_output_LB_10h_1	7,719,745	7,468,495 **(96.8)**	3,432,728	3,141,388	2,851,451 **(90.8)**	190,806	153,486 **(80.4)**
P125109_output_LB_10h_2	12,018,716	11,730,618 **(97.6)**	4,645,517	4,293,107	3,859,904 **(89.9)**		
P125109_output_MAC_1	14,185,462	13,953,695 **(98.4)**	4,108,123	3,700,162	3,329,720 **(90.0)**	176,921	142,198 **(80.4)**
P125109_output_MAC_2	6,691,075	6,411,282 **(95.8)**	3,066,055	2,768,978	2,510,327 **(90.7)**		
D7795_input_LB_1	3,338,608	3,272,795 **(98.0)**	2,117,787	2,041,159	1,830,339 **(89.7)**	137,841	110,118 **(79.9)**
D7795_input_LB_2	2,759,938	2,615,176 **(94.8)**	2,031,135	1,871,948	1,675,040 **(89.5)**		
D7795_output_LB_10h_1	3,442,438	3,267,108 **(94.9)**	2,319,531	2,123,585	1,898,162 **(89.4)**	131,309	105,051 **(80.0)**
D7795_output_LB_10h_2	3,622,456	3,529,827 **(97.4)**	2,203,783	2,090,960	1,844,384 **(88.2)**		
D7795_output_MAC_1	2,711,255	2,651,138 **(97.8)**	1,474,023	1,398,216	1,232,219 **(88.1)**	117,328	93,731 **(79.9)**
D7795_output_MAC_2	4,062,876	3,953,627 **(97.3)**	2,377,824	2,237,906	1,968,539 **(88.0)**		

[a] Number of unique insertions for each strain and condition was calculated by combining reads from the appropriate biological replicates.

5.2.3 Identification of P125109 genes with differential fitness in macrophage infection

Genes affecting *Salmonella* intracellular survival and growth in RAW 264.7 macrophages were identified by comparing the macrophage output samples with the input samples and calculating the changes in frequency of reads mapped to each gene, expressed as $\log_2$(fold change) (FC) using the Hinton Laboratory's published approach (Canals *et al.*, 2019a). A gene is considered to exhibit differential fitness if its $\log_2$FC value is less than 1 (attenuated fitness) or greater than 1 (increased fitness) and has a *P*-value < 0.05. Genes affecting growth in LB for 10 h were identified in the same fashion, by comparing the LB output samples with the input samples. Required genes were identified from the input sample using the insertion index (described in Chapter 4) and excluded from the list of differential fitness genes.

Figure 5.3 shows the overall changes in abundance of the P125109 transposon mutants following 12-h macrophage infection. A total of 479 required genes was identified from essentiality analysis of the input library (**Appendix 8**). Transposon insertions in 327 genes were associated with attenuation of replication within macrophages (**Appendix 9**).

The macrophage output library was extracted from transposon mutants grown for 10 h in LB following release from the macrophages (**Figure 5.1**). As demonstrated in Chapter 4 and related TIS screens of *Salmonella* mutants following passages in LB (Canals *et al.*, 2019a; Khatiwara *et al.*, 2012; Langridge *et al.*, 2009), some transposon mutants will be lost as a result of the LB passage. To identify genes that are required for fitness in intra-macrophage replication and not associated with growth in general, the 327 macrophage-attenuated fitness mutants were first compared with the mutants that showed reduced fitness after growth in LB for 10 h, identifying 100 genes that had reduced fitness in LB growth (**Figure 5.4A**). The resulting 227 genes

were further cross-referenced with the genes required for *in vitro* growth under laboratory conditions (Chapter 4), identifying 177 “macrophage-specific” genes that showed attenuation in intra-macrophage replication and did not impact upon growth *in vitro* (**Figure 5.4B**), and a total of 143 “macrophage-associated” genes (numbers in white text in **Figure 5.4**) that showed both attenuation in macrophage infection and *in vitro* growth were identified. The terms “macrophage-specific” and “macrophage-associated” have been defined previously (Canals *et al.*, 2019a).

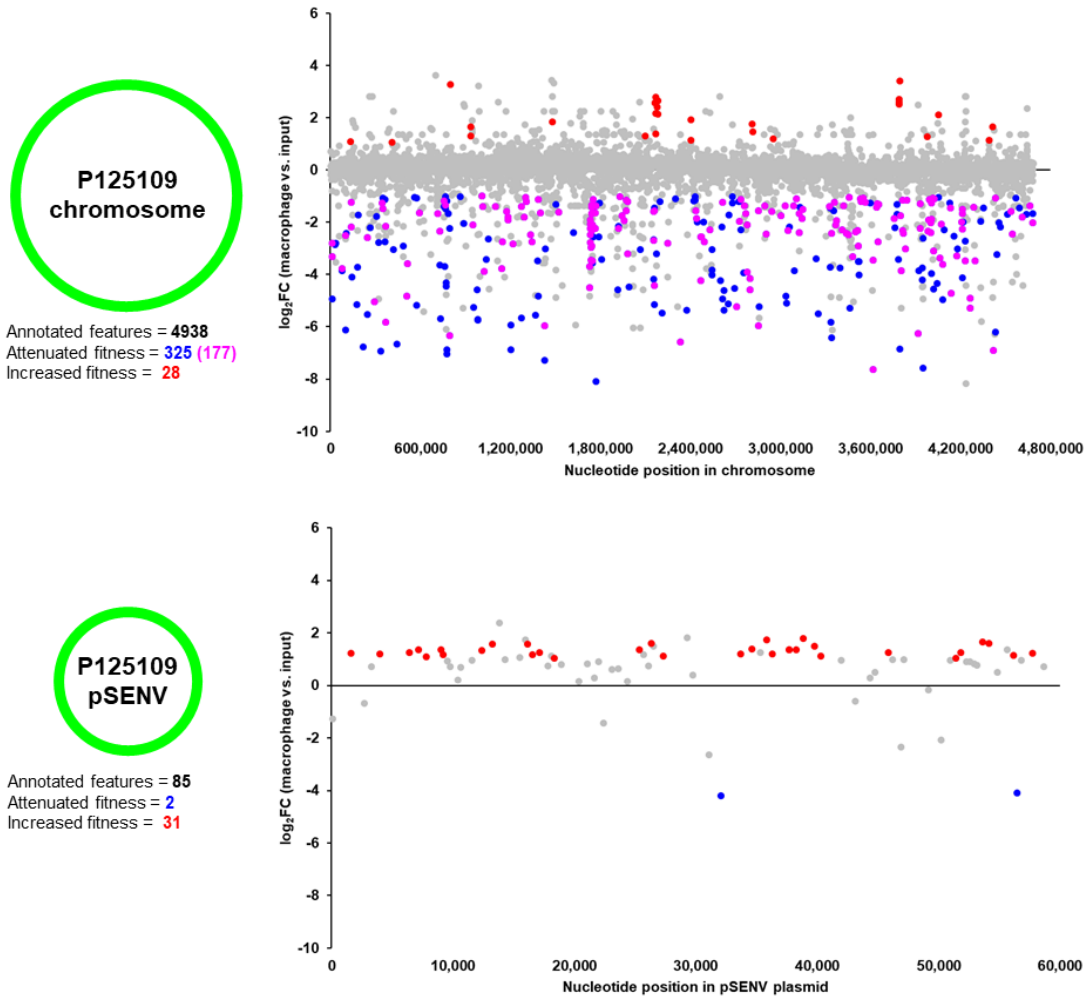

Figure 5.3 Changes in frequency of *S*. Enteritidis P125109 mutants after screening in RAW 264.7 macrophages

The P125109 transposon library was used to infect RAW 264.7 macrophages for 12 h. Relative abundance of each mutant was determined by comparing the frequency of sequenced reads mapped to each gene after the infection (macrophage output) to the initial inoculum (input). Each position on the *x*-axis represents the starting nucleotide position of each gene locus on the P125109 chromosome (top panel) and pSENV plasmid (bottom panel), and the *y*-axis represents the $\log_2$(fold change) of changes in abundance of mapped reads. "Annotated Features" refers to a combination of protein-coding genes and sRNA genes (Section 4.2.2.1). Loci with significant changes in abundance ($P < 0.05$) are shown in blue (attenuated fitness) or red (increased fitness), and loci with $P \geq 0.05$ are shown in grey. "Macrophage-specific" attenuated loci are shown in magenta (see text in Section 5.2.3 for explanation).

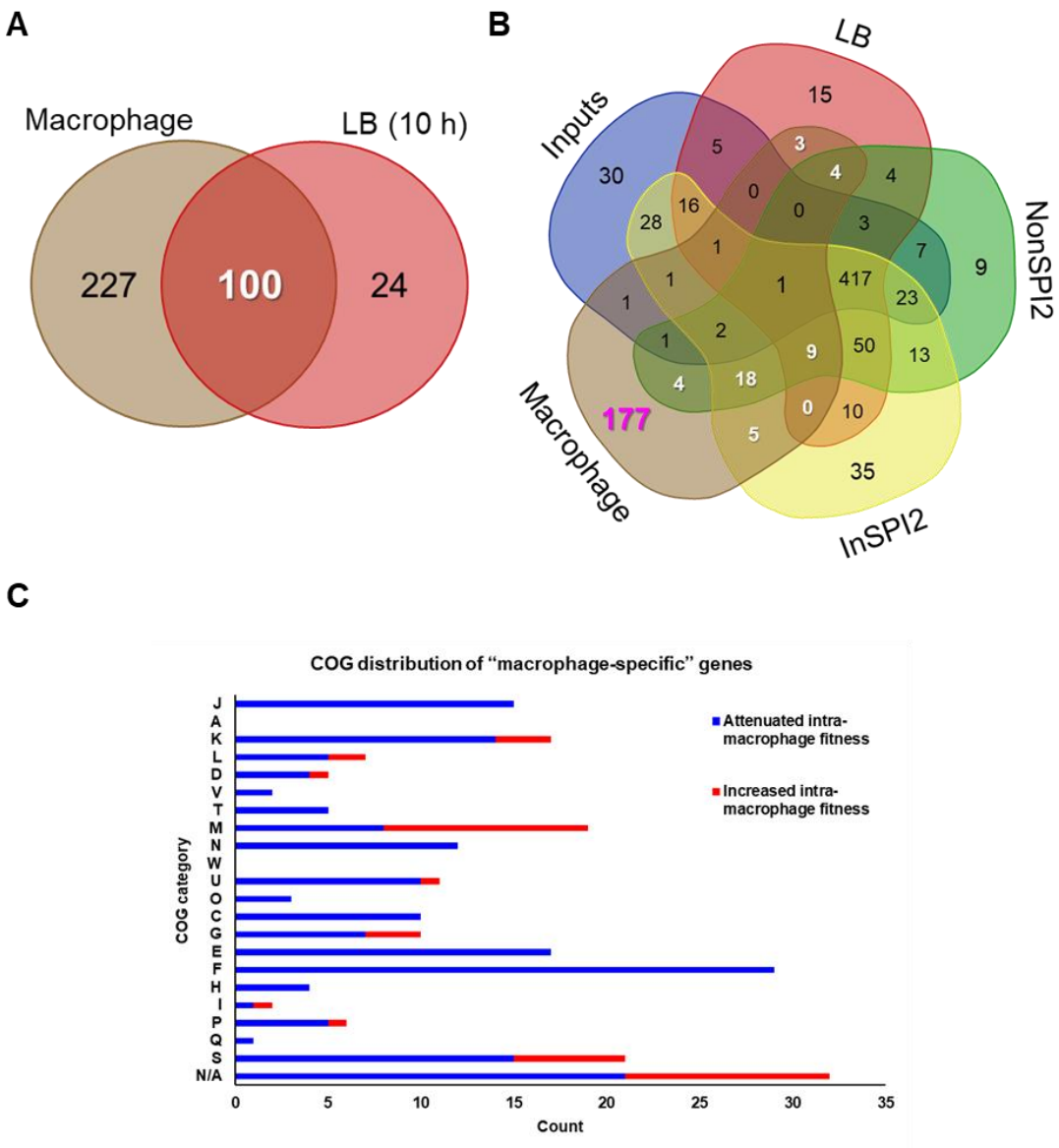

Figure 5.4 Identification of *S.* Enteritidis P125109 genes that cause fitness alteration in macrophages

A. Venn diagram comparing the genes with a $\log_2$ reduction (i.e. two-fold decrease) in number of mapped reads in output pools following infection in macrophage for 12 h or culture in LB for 10 h, compared with the input. The gene lists are available in **Appendix 10**. **B.** Comparison of the 227 macrophage-attenuated genes from panel **A** with genes identified as required for *in vitro* growth under different laboratory conditions described in Chapter 4. The inputs in panel **B** refer to the combined required gene sets identified from the input libraries described in both Chapter 4 and this chapter. The various gene lists are available in **Appendix 10**. Number of "macrophage-specific" genes is highlighted in magenta and "macrophage-associated" genes is highlighted in white (see text for definition of the two categories). **C.** COG categories distribution of the "macrophage-specific" genes whose mutants had attenuated or increased fitness. Definitions of COG categories are given in **Table 4.3**. Categories A and W have been omitted as no COGs from these two categories were associated with the "macrophage-specific" genes. Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/. N/A, not assigned.

The 177 “macrophage-specific” genes included many well-characterised genes important for *Salmonella* intra-macrophage survival, such as 22 genes from SPI-2, *mgtC* from SPI-3, and global regulatory systems that control *Salmonella* virulence (e.g. *ompR*, *phoQ*, *ssrB*). Two SPI-1 genes, *hilC* and *iagB*, were also identified. There were 74 genes related to various metabolic processes, including arginine biosynthesis (*arg* genes), histidine biosynthesis (*his*) genes, the TCA cycle (*sucD*) and oxidative phosphorylation (*ndh*), reflecting the nutritional stresses encountered by the bacteria within the macrophage environment. Three P125109-specific genes were also identified as “macrophage-specific”, namely *SEN0912* (encoding a hypothetical protein) and two tRNA genes (*tRNA-Ala* and *tRNA-Thr*). No genes from the ROD9 region were identified, despite reports confirming a role in virulence of several genes in macrophage and animal infection (Das *et al.*, 2018, Das *et al.*, 2020; Silva *et al.*, 2012). Based on COG classifications (see Chapter 4), the majority of the 177 genes were associated with nucleotide metabolism (F, 17%), amino acid metabolism (E, 10%), transcription (K, 8%) and translation (J, 9%).

Transposon insertion in 61 genes resulted in increased fitness in macrophage infection ($\log_2$FC > −1, P < 0.05). “Macrophage-specific” and “macrophage-associated” genes were identified as described previously, by comparing the genes that showed increased fitness in intra-macrophage infection and increased fitness after 10-h growth in LB. Forty genes were found to be “macrophage-specific” (**Appendix 9**). Among the 40 genes were 12 genes related to LPS O-antigen biosynthesis (*rfa* and *rfb* genes) and the sRNA PinT. The increased intra-macrophage fitness conferred by transposon disruption in the *rfa*/*rfb* genes was also observed in *S.* Typhimurium infection of RAW 264.7 macrophages (Canals *et al.*, 2019a; Venturini *et al.*, 2020). Similarly, Kim *et al.* (2019) reported that a deletion in PinT causes increased fitness of *S.* Typhimurium in systemic infection of mice.

5.2.4 Identification of D7795 genes with differential fitness in macrophage infection

Genes important for intra-macrophage replication of D7795 in RAW 264.7 macrophages were identified as described in the previous section. Essentiality analysis of the input sample identified 432 required genes (**Appendix 8**), and these genes were excluded from the list of genes that caused differential fitness in macrophage infection.

Figure 5.5 shows the overall changes in abundance of the D7795 transposon mutants following 12-h macrophage infection. Transposon insertions in 329 genes caused attenuation ($\log_2$FC < −1, P < 0.05) during RAW macrophage infection (**Appendix 11**). “Macrophage-specific” and “macrophage-associated” genes were identified as described previously. Of the 329 genes, 78 genes exhibited reduced fitness during growth in LB for 10 h. Cross-referencing the 251 genes with the genes required for *in vitro* growth under laboratory conditions (Chapter 4) identified 201 “macrophage-specific” genes and 123 “macrophage-associated” genes (**Figure 5.6**, **Appendix 11**).

Similar to the findings for P125109, the 201 D7795 “macrophage-specific” genes are primarily found in the SPI regions. Majority of the attenuated mutants were located in SPI-2; several others were identified in SPI-3 (*SEN3578*, *amgR*, *mgtC*), SPI-4 (*siiF*), SPI-5 (*pipC*), SPI-6 (*SEN0286*) and SPI-10 (*SEN4249*). *SEN1008* from ROD9 (SPI-19) was also identified as attenuated. Distribution of the COG functional categories of the “macrophage-specific” genes is broadly similar to that in P125109, with nucleotide metabolism (F) and amino acid metabolism (E) genes forming the largest categories.

Transposon insertion in 18 genes resulted in increased fitness in the macrophage environment ($log_2FC > 1$, $P < 0.05$); 3 genes are encoded on the smaller pRGI00316 plasmid and have no orthologues in P125109 (or *S.* Typhimurium LT2 and D23580). Of the remaining 15 genes, 13 genes are involved in LPS biosynthesis.

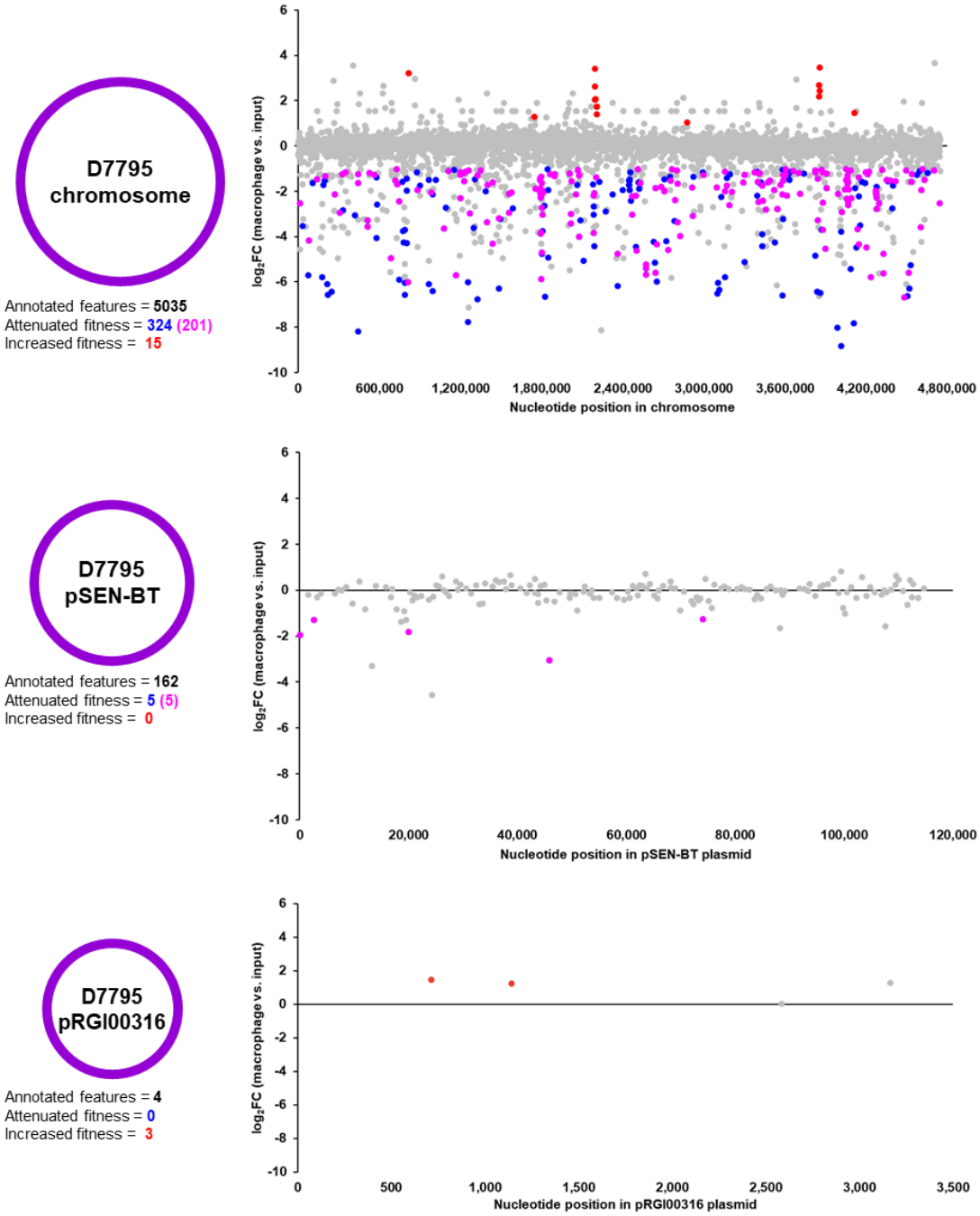

Figure 5.5 Changes in frequency of *S*. Enteritidis D7795 mutants after screening in RAW 264.7 macrophages

The D7795 transposon library was used to infect RAW 264.7 macrophages for 12 h. Relative abundance of each mutant was determined by comparing the frequency of sequenced reads mapped to each gene after the infection (macrophage output) to the initial inoculum (input). Each position on the *x*-axis represents the starting nucleotide position of each gene locus on the D7795 chromosome (top), pSEN-BT (middle) and pRGI00316 plasmids (bottom), and the-*y* axis represents the $\log_2$(fold change) of changes in abundance of mapped reads. "Annotated Features" refers to a combination of protein-coding genes and sRNA genes (Section 4.2.2.1). Loci with significant changes in abundance ($P < 0.05$) are shown in blue (attenuated fitness) or red (increased fitness), and loci with $P \geq 0.05$ are shown in grey. "Macrophage-specific" attenuated loci are shown in magenta.

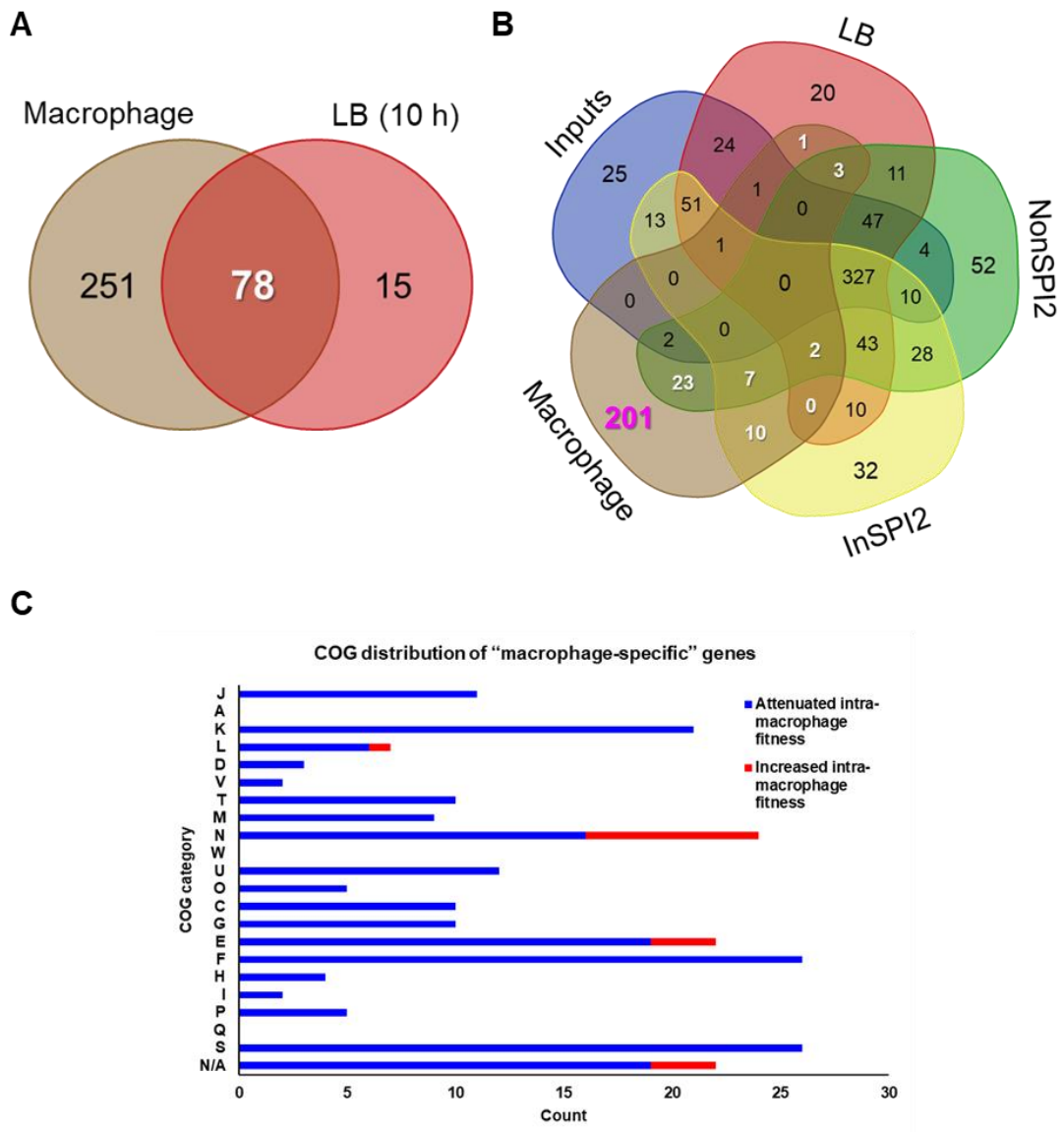

Figure 5.6 Identification of *S.* Enteritidis D7795 genes that cause fitness alteration in macrophages

A. Venn diagram indicating the union of genes with greater than two-fold reduction in mapped reads in the output pools following culture in LB broth for 10 h or in macrophage for 12 h compared with the input. The gene lists are available in **Appendix 12**. **B.** Comparison of the 251 macrophage-attenuated genes from panel **A** with genes identified as required for *in vitro* growth under different laboratory conditions described in Chapter 4. The inputs in panel **B** refer to the combined required gene sets identified from the input libraries described in both Chapter 4 and this chapter. The various gene lists are available in **Appendix 12**. Number of "macrophage-specific" genes is highlighted in magenta and "macrophage-associated" genes is highlighted in white. **C.** COG categories distribution of the "macrophage-specific" genes whose mutants had attenuated or increased fitness. Definitions of COG categories are given in **Table 4.3**. Categories A, W and Q have been omitted as there were no COGs associated with the two categories in the "macrophage-specific" genes. Venn diagram was generated using http://bioinformatics.psb.ugent.be/webtools/Venn/. N/A, not assigned

5.2.5 Comparing *S.* Enteritidis genes displaying differential fitness in macrophage infection with African *S.* Typhimurium D23580

P125109 or D7795 genes identified as displaying differential fitness in macrophage infection were compared with the gene requirements for macrophage infection published in the Hinton Laboratory's recent TIS-based study on African *S.* Typhimurium D23580 (Canals *et al.*, 2019a). To ensure consistency, the sequencing data from the Canals *et al.* (2019a) macrophage infection experiments was re-processed and analysed using the same bioinformatic pipelines described in Section 2.8.4. **Appendix 13** shows the 3975 orthologous genes shared between P125109, D7795 and D23580, and the $\log_2$FC values associated with each gene following a single passage through macrophages. After excluding required genes based on analysis of input libraries for each strain, the number of genes that showed attenuation in macrophage infection following transposon insertion was 307 in P125109, 311 in D7795 and 217 in D23580 respectively; these genes include both "macrophage-specific" and "macrophage-associated" genes. My datasets contained an additional "output_LB_10h" dataset that was absent in the Canals *et al.* (2019a) study, which may identify a greater number of "macrophage-associated" genes in *S.* Enteritidis; therefore to ensure consistency, the "macrophage-associated" genes were not removed.

Comparing the three gene lists showed that 147 genes were attenuated in macrophage infection for all three strains, and there are more genes common to both *S.* Enteritidis strains (70) than that shared between P125109 and D23580 (20), or between D7795 and D23580 (22) (**Figure 5.7**, **Appendix 13**). The 147 genes also showed significant overlap with genes required for *Salmonella* systemic infection in mice identified in three other genome-wide studies: 44 genes in common with Silva *et al.* (2012) (microarray-based screen of *S.* Enteritidis P125109 Tn*5* library); 85 genes in common with Chaudhuri *et al.* (2013) (TraDIS with *S.* Typhimurium SL1344);

and 47 genes in common with Silva-Valenzuela *et al*. (2016) (defined single gene deletion library of *S.* Typhimurium 14028) (**Appendix 13E**).

Transposon insertion in 13 genes was shown to increase intra-macrophage fitness of all three *Salmonella* strains (**Appendix 13L**). As discussed previously, majority of the genes that increased intra-macrophage fitness were the *rfa*/*rfb* genes. A sRNA STnc1320 was also consistently identified as enhancing intra-macrophage fitness, although it should be noted that STnc1320 overlaps the *rfbD* gene.

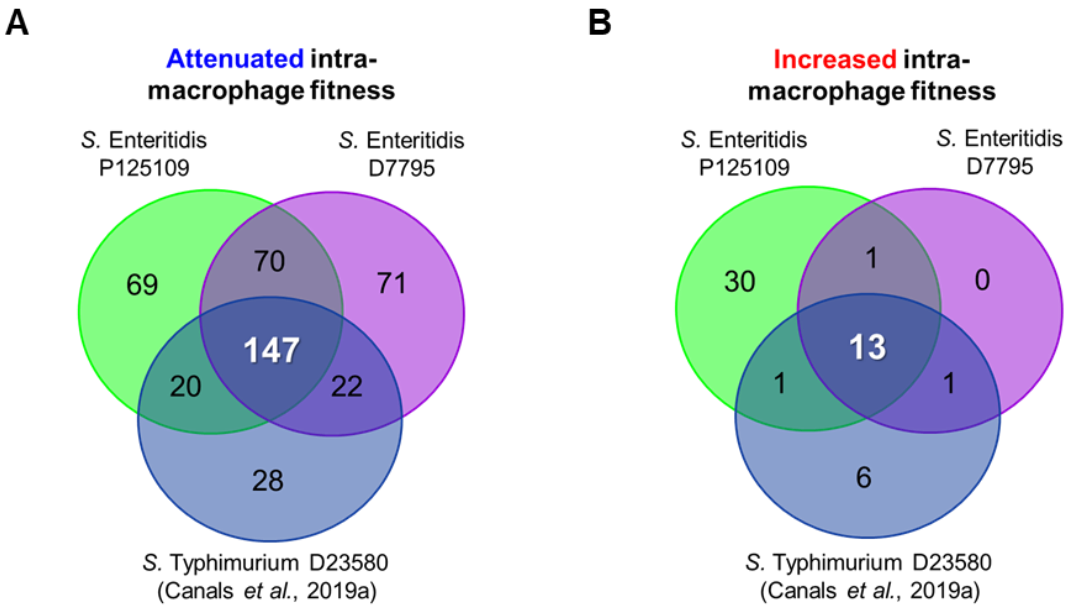

Figure 5.7 Comparison of *S.* Enteritidis P125109, *S.* Enteritidis D7795 and *S.* Typhimurium D23580 genes that cause fitness alteration in macrophage infection

Venn diagrams comparing (**A**) genes with a $\log_2$FC < −1 (P < 0.05) and (**B**) $\log_2$FC > 1 (P < 0.05) in number of mapped reads in output pools following infection in macrophage for 12 h compared with the input. Data for *S.* Typhimurium D23580 were obtained from the Canals *et al.* (2019a) study and re-analysed using the same bioinformatic pipeline described in Section 2.8.4. The various gene lists are available in **Appendix 13**.

5.2.5.1 SPI-1 to SPI-5 genes

Figure 5.8 shows the distribution of genes in the five major SPI regions and their associated log_2FC values. As expected, the SPI-2 region is important for macrophage replication of all three *Salmonella* strains, with majority of transposon insertions in genes encoding structural components of the TTSS showing high degrees of attenuation. Interestingly, *orf245* (encoding a hypothetical cytoplasmic protein) is important for intra-macrophage replication of the two *S.* Enteritidis strains, but in *S.* Typhimurium D23580 is designated as an essential gene. *mgtC* (SPI-3) is important for intra-macrophage fitness of P125109, D7795 and *S.* Typhimurium D23580, although the level of attenuation is lower in the two *S.* Enteritidis strains (P125109 log_2FC = −1.15, $P = 1.8 \times 10^{-18}$; D7795 log_2FC = −1.87, $P = 4.7 \times 10^{-40}$) compared to D23580 (log_2FC = −2.27, $P = 4.8 \times 10^{-40}$). Genes in the other SPI regions display a more varied pattern e.g. *iagB* (SPI-1) and *pipB* (SPI-5) mutants were significantly attenuated in P125109 only, while *siiB* and *siiF* were significantly attenuated in both *S.* Enteritidis strains but not in *S.* Typhimurium D23580.

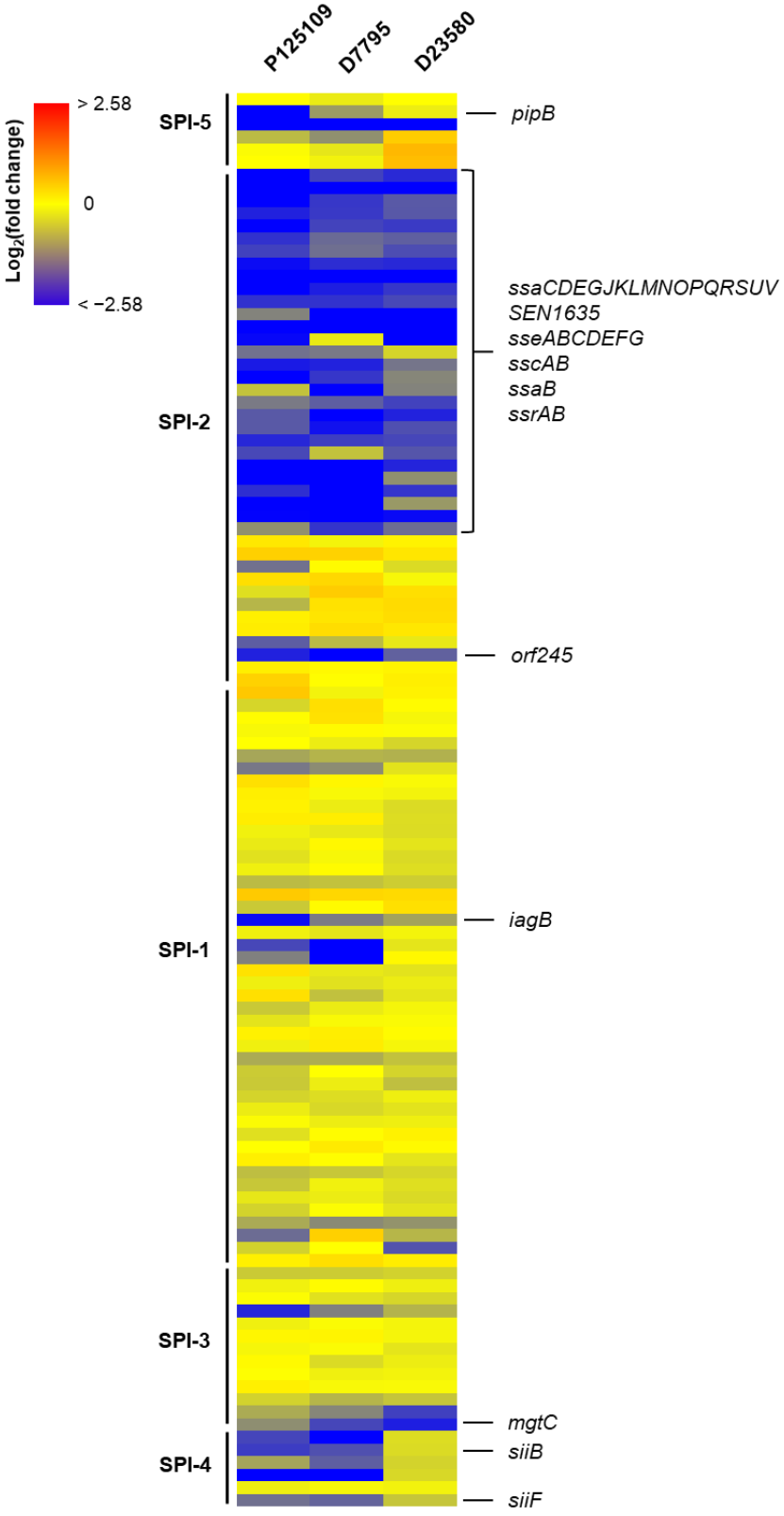

Figure 5.8 Comparison of SPI-1 to SPI-5 genes in *S.* Enteritidis P125109, *S.* Enteritidis D7795 and *S.* Typhimurium D23580 that contribute to fitness in macrophage infection

Heat map representing log_2FC (macrophage output vs. input) values associated with each gene. Statistical significance is not indicated; refer to **Appendix 13E** for actual log_2FC and *P*-values.

5.2.5.2 Effector proteins

Effector proteins are small molecules secreted by a pathogen to modulate host cell activity and aid the infection process (Jennings *et al.*, 2017; Johnson *et al.*, 2018). *Salmonella* effector proteins are secreted by two TTSS encoded on SPI-1 and SPI-2 during different stages of the infection process, with TTSS-1 primarily associated with invasion of host cells and TTSS-2 mainly for intracellular survival (dos Santos *et al.*, 2018). The effect of transposon insertions in 38 genes encoding known and predicted effector proteins in *S.* Enteritidis P125109, D7795 and *S.* Typhimurium D23580 was determined, and showed that mutations in 5, 6 and 2 of the 38 genes, respectively, caused intra-macrophage attenuation. Only a small number of effectors exhibited differential fitness in macrophage infection (**Table 5.2**).

Table 5.2 Effect of transposon insertion in genes encoding effector proteins on *S.* Enteritidis P125109, *S.* Enteritidis D7795 and *S.* Typhimurium D23580 fitness during macrophage infection

Secreted by	Name	P125109 locus tag	D7795 locus tag	D23580 locus tag	P125109 log_2FC	D7795 log_2FC	D23580 log_2FC
SPI-1	SopB	P125109_01071	D7795_01154	STMMW_11031	−0.04	−0.25	0.73
	SopE2	P125109_01333	D7795_01414	STMMW_18441	−0.66	−1.63	**1.76***
	SopF	P125109_02049	D7795_02187	STMMW_12491	−0.02	**−1.21***	0.40
	SopA	P125109_02346	D7795_02393	STMMW_20971	0.06	−0.06	0.11
	SptP	P125109_03092	D7795_03190	STMMW_28401	−0.15	−0.26	−0.11
	SipA	P125109_03096	D7795_03194	STMMW_28441	−0.16	−0.30	−0.19
	SipD	P125109_03098	D7795_03196	STMMW_28451	−0.55	−0.20	−0.10
	SipC	P125109_03099	D7795_03197	STMMW_28461	−0.26	−0.05	−0.04
	SipB	P125109_03100	D7795_03198	STMMW_28471	0.15	0.18	0.04
	SopD	P125109_03165	D7795_03263	STMMW_29081	−0.87	−0.98	−0.38
SPI-1 or SPI-2	SlrP	P125109_00839	D7795_00864	STMMW_08521	−0.21	−0.08	0.01
	GtgE	P125109_01034	D7795_01117	STMMW_10681	0.08	−0.57	−0.35
	SteA	P125109_01661	D7795_01747	STMMW_15781	0.02	−0.02	−0.56
	AvrA	P125109_03073	D7795_03171	STMMW_28271	0.01	−0.19	−0.42
	SpvD	P125109_05007	D7795_05101	SLT-BT0211	−2.09	**−3.05***	**1.18***
SPI-2	SopD2	P125109_00989	D7795_01014	STMMW_09831	−0.43	**−2.06***	0.06
	PipB	P125109_01068	D7795_01151	STMMW_11001	**−2.65***	−1.01	−0.18
	GtgA	P125109_01286	D7795_01369	STMMW_10381	−0.15	−0.09	−0.01
	SteC	P125109_01508	D7795_01589	STMMW_16931	0.20	−1.51	0.03
	SseJ	P125109_01605	D7795_01689	STMMW_16261	−0.86	−0.76	−0.37
	SseG	P125109_01848	D7795_01934	STMMW_14101	**−1.44***	**−1.36***	−0.42
	SseF	P125109_01849	D7795_01935	STMMW_14091	**−2.32***	**−2.23***	**−1.40***
	SsaB	P125109_01860	D7795_01946	STMMW_13981	**−3.47***	−2.82	−1.03
	SifA	P125109_02065	D7795_02203	STMMW_12331	**−1.03***	**−1.57***	**−1.64***
	SEN2134	P125109_02425	D7795_02473	STMMW_21701	0.09	−0.11	−0.13
	SspH2	P125109_02527	D7795_02575	STMMW_22721	0.15	0.30	0.19
	SseL	P125109_02578	D7795_02626	STMMW_23111	−0.72	−0.52	−0.63
	PipB2	P125109_02976	D7795_03070	STMMW_27461	0.85	0.33	0.04
	CigR	P125109_04062	D7795_04161	STMMW_37511	0.16	−0.07	−0.05
	SseK1	P125109_04485	D7795_04581	STMMW_41101	−0.20	−0.43	−0.77
	SpvC	P125109_05006	D7795_05102	SLT-BT0221	−0.19	−0.50	0.52
	SpvB	P125109_05005	D7795_05103	SLT-BT0231	0.99	−0.09	0.76
Unknown	SEN0342	P125109_00387	D7795_00390	STMMW_04291	0.36	−0.33	−0.28
	PipA	P125109_01067	D7795_01150	STMMW_10991	0.04	−0.19	0.01
	YdgH	P125109_01770	D7795_01856	STMMW_14781	0.01	−0.47	0.50
Candidate^	SEN1228	P125109_01384	D7795_01465	STMMW_18011	0.41	−1.30	0.26
	PdgL	P125109_01644	D7795_01729	STMMW_15941	0.68	−0.14	0.32
	YiiQ	P125109_04401	D7795_04498	STMMW_40471	0.21	−0.10	−0.27

Log_2FC values marked with asterisk (*) and in bold indicate they met the criteria of $log_2FC < -1$ or > 1 and *P*-value < 0.05; colour scale is the same as the one shown in **Figure 5.8**. Effector protein names are shown in pink if fitness effect differs across the three strains, and blue if the fitness effect is similar. Table of effector proteins was compiled by Dr Alex Predeus and Yan Li; candidate (^) effector proteins were proposed by the Niemann *et al.* (2011) study.

5.2.5.3 *S.* Enteritidis-common genes with attenuated intra-macrophage fitness

A total of 70 genes that were associated with attenuated intra-macrophage fitness for both P125109 and D7795 were identified in the three-way comparison between both *S.* Enteritidis strains and *S.* Typhimurium D23580 (**Figure 5.7, Appendix 13F**). Because these 70 genes were only found in the *S.* Enteritidis strains, and not in *S.* Typhimurium D23580, they were classified as "*S.* Enteritidis-common" genes. Of the 70 genes, 38 genes have been shown to be required for optimal infection in one other *Salmonella* virulence study (Chaudhuri *et al.*, 2013; Silva-Valenzuela *et al.*, 2016; Silva *et al.*, 2012). The remaining 32 genes represent candidates for *S.* Enteritidis-specific genes required for macrophage fitness (**Table 5.3**).

Further investigation revealed that several genes had been identified in other genetic screens or experimentally verified to play a virulence role in other *Salmonella* serovars. For example, Husna *et al.* (2019) reported that the virulence of *S.* Typhimurium Δ*pfs* was attenuated during murine infection, and the attenuation can be attributed to the toxic accumulation of S-adenosylhomocysteine (SAH); *ybiS* was negatively selected in a *S.* Typhi transposon library screen in humanised mice (Karlinsey *et al.*, 2019). It should be noted that the comparison in **Figure 5.7** does not take into account *S.* Enteritidis genes that are absent in *S.* Typhimurium genomes e.g. RODs (Thomson *et al.*, 2008), as only orthologues were used in the comparison.

Table 5.3 The 32 *S.* Enteritidis-common genes that contribute to fitness during macrophage infection and were not identified in Silva *et al.* (2012), Chaudhuri *et al.* (2013) or Silva-Valenzuela *et al.* (2016)

Name	Product	SEN identifier	P125109 locus tag	D7795 locus tag	D23580 locus tag	P125109 log_2FC	D7795 log_2FC	D23580 log_2FC
aceE	pyruvate dehydrogenase E1 component	SEN0156	P125109_00177	D7795_00178	STMMW_01581	−5.16	−5.80	Required
pfs	MTA/SAH nucleosidase	SEN0212	P125109_00237	D7795_00238	STMMW_02131	−5.52	−6.42	Required
rnhA	Ribonuclease H	SEN0265	P125109_00299	D7795_00300	STMMW_02621	−1.79	−2.96	−0.70
purK	phosphoribosylaminoimidazole carboxylase ATPase subunit	SEN0514	P125109_00577	D7795_00584	STMMW_06011	−1.08	−1.38	−0.68
ybiS	putative exported protein	SEN0782	P125109_00882	D7795_00907	STMMW_08881	−1.02	−1.17	−0.35
cmk	cytidylate kinase	SEN0884	P125109_00997	D7795_01022	STMMW_09911	−5.75	−6.42	Required
motA	motility protein A	SEN1080	P125109_01216	D7795_01299	STMMW_19061	−1.59	−1.55	−0.97
cls	Cardiolipin synthetase	SEN1295	P125109_01459	D7795_01540	STMMW_17341	−3.49	−1.61	−0.76
yciB	Probable intracellular septation protein	SEN1299	P125109_01465	D7795_01546	STMMW_17301	−1.13	−4.31	−0.98
orf245	Orf 245 protein	SEN1664	P125109_01872	D7795_01958	STMMW_13861	−2.25	−2.65	Required
lpp	major outer membrane lipoprotein	SEN1668	P125109_01877	D7795_01963	STMMW_13822	−8.09	−6.64	−5.82 (ns)
flgN	flagella synthesis protein FlgN	SEN1877	P125109_02127	D7795_02265	STMMW_11791	−1.22	−1.30	−0.81
wcaE	glycosyltransferase	SEN2107	P125109_02395	D7795_02442	STMMW_21431	−1.22	−1.60	−0.76
crr	pts system%2C glucose-specific IIA component	SEN2414	P125109_02744	D7795_02792	STMMW_24521	−3.06	−5.24	Required
SEN2554	putative transmembrane transport protein	SEN2554	P125109_02892	D7795_02940	STMMW_25901	−1.22	−1.36	−0.54
corE	putative membrane protein	SEN2599	P125109_02946	D7795_02994	STMMW_26921	−1.66	−1.08	−0.73
yfjF	conserved hypothetical protein	SEN2606	P125109_02954	D7795_03002	STMMW_27001	−2.11	−1.67	−0.48
pepP	proline aminopeptidase II	SEN2901	P125109_03300	D7795_03397	STMMW_30181	−1.10	−1.75	−0.90
SEN2923	conserved hypothetical protein	SEN2923	P125109_03328	D7795_03424	STMMW_30411	−1.42	−1.25	−0.96
yrfI	heat shock protein	SEN3324	P125109_03780	D7795_03879	STMMW_34881	−1.10	−1.29	−0.63

Table 5.3 *(continued)* The 32 *S.* Enteritidis-common genes that contribute to fitness during macrophage infection and were not identified in Silva *et al.* (2012), Chaudhuri *et al.* (2013) or Silva-Valenzuela *et al.* (2016)

Name	Product	SEN identifier	P125109 locus tag	D7795 locus tag	D23580 locus tag	P125109 log_2FC	D7795 log_2FC	D23580 log_2FC
spoT	guanosine-3'%2C5'-bis(diphosphate) 3'-pyrophosphohydrolase%3B CG Site No. 156	SEN3564	P125109_04038	D7795_04137	STMMW_37311	−2.32	−1.16	−0.80
gppA	guanosine-5'-triphosphate%2C3'-diphosphate pyrophosphatase (guanosine pentaphosphatase)	SEN3719	P125109_04220	D7795_04318	STMMW_38891	−1.13	−1.02	−0.86
yigP	conserved hypothetical protein	SEN3766	P125109_04280	D7795_04378	STMMW_39421	−4.34	−7.84	−1.83 (ns)
yshA	conserved hypothetical protein	SEN3803	P125109_04328	D7795_04426	STMMW_39801	−2.28	−3.50	−0.70
SEN3842	conserved hypothetical protein	SEN3842	P125109_04370	D7795_04467	STMMW_40161	−1.26	−1.64	−0.85
sodA	manganese superoxide dismutase	SEN3845	P125109_04373	D7795_04470	STMMW_40201	−1.45	−1.17	−0.41
oxyR	hydrogen peroxide-inducible regulon activator	SEN3919	P125109_04451	D7795_04547	STMMW_40891	−4.11	−2.77	−0.97
pgi	glucose-6-phosphate isomerase	SEN3987	P125109_04532	D7795_04628	STMMW_41701	−3.47	−1.54	−0.78
siiB	putative integral membrane protein	SEN4027	P125109_04576	D7795_04672	STMMW_42091	−1.97	−1.76	−0.36
siiF	putative type-1 secretion protein	SEN4032	P125109_04580	D7795_04676	STMMW_42131	−1.45	−1.56	−0.58
SEN4091	putative exported protein	SEN4091	P125109_04643	D7795_04739	STMMW_42692	−6.91	−6.68	−0.80
hilE	conserved hypothetical protein	SEN4270	P125109_04841	D7795_04937	STMMW_44551	−1.47	−1.19	−0.54

All D7795 and P125109 genes shown have log_2FC < −1 and *P*-value < 0.05. D23580 genes designated required based on essentiality analysis of input. Colour scale is the same as shown in **Figure 5.8**. ns, non-significant (*P*-value ≥ 0.05).

5.2.5.4 Invasive African *Salmonella*-common genes with attenuated intra-macrophage fitness

Twenty-two genes were identified that were shared by both African *S.* Enteritidis D7795 and African *S.* Typhimurium D23580 and designated as "Invasive African *Salmonella*-common genes" (**Figure 5.7**, **Appendix 13G**). Of the 22 genes, 7 genes were not identified in Silva *et al.* (2012), Chaudhuri *et al.* (2013) and Silva-Valenzuela *et al.* (2016) as important for *Salmonella* virulence (**Table 5.4**). Four genes, namely *corA*, *glnA*, *amgR* and *ptsN,* have already been assigned a role in *Salmonella* virulence (Choi *et al.*, 2010; Klose and Mekalanos, 1997; Lee and Groisman, 2010; Papp-Wallace *et al.*, 2008). The remaining three genes, *folK*, *aspC* and *nuoI*, are involved in key metabolic processes such as amino acid biosynthesis and oxidative phosphorylation, and so could play a hitherto unrecognised role in virulence of other *Salmonella* serovars.

Taken together, the findings of this section suggest that no novel virulence factors involved in intra-macrophage replication are shared by the two invasive *Salmonella* strains.

Table 5.4 Genes common to African *S.* Enteritidis D7795 and African *S.* Typhimurium D23580 that contribute to fitness during macrophage infection and that were not identified by Silva *et al.* (2012), Chaudhuri *et al.* (2013) and Silva-Valenzuela *et al.* (2016)

Gene	Product	SEN identifier	D7795 locus tag	D23580 locus tag	D7795 log_2FC	D23580 log_2FC
folK	2-amino-4-hydroxy-6-hydroxymethyldihydropteridinepyrophosphokinase	SEN0188	D7795_00211	STMMW_01891	−6.11	−1.27
aspC	aspartate aminotransferase	SEN0902	D7795_01041	STMMW_10091	−1.49	−1.27
nuoI	NADH dehydrogenase I chain I	SEN2303	D7795_02662	STMMW_23431	−1.47	−1.65
ptsN	nitrogen regulatory IIA protein	SEN3155	D7795_03690	STMMW_33211	−4.43	−2.62
amgR	NONE	NONE	D7795_04165	STMMW_ncRNA_247	−1.22	−1.93
corA	magnesium and cobalt transport protein	SEN3749	D7795_04359	STMMW_39231	−2.32	−1.44
glnA	glutamine synthetase	SEN3795	D7795_04417	STMMW_39711	−2.22	−2.38

All values shown have *P*-value < 0.05. Colour scale is the same as shown in **Figure 5.8**.

5.2.5.5 Candidate novel P125109- or D7795-only virulence genes

In **Figure 5.7**, 69 and 71 genes in P125109 and D7795 respectively were identified as exhibiting attenuated intra-macrophage fitness. By performing the series of comparisons illustrated in **Figure 5.9**, 22 P125109-only genes and 39 D7795-only "macrophage-specific" genes with attenuated fitness were identified (**Table 5.5** and **Table 5.6**). Some genes are uniquely present in one strain, while others have orthologues in the other *S.* Enteritidis strain, *S.* Typhimurium D23580 and LT2, but did not show attenuated fitness during macrophage infection (an example highlighted earlier was differences in fitness of *sopD2* mutant). The genes shown in **Table 5.5** and **Table 5.6** represent candidate novel virulence genes for P125109 and D7795 respectively. Experimental validation of these candidates by individual gene deletion mutants is necessary to verify their role in intra-macrophage replication.

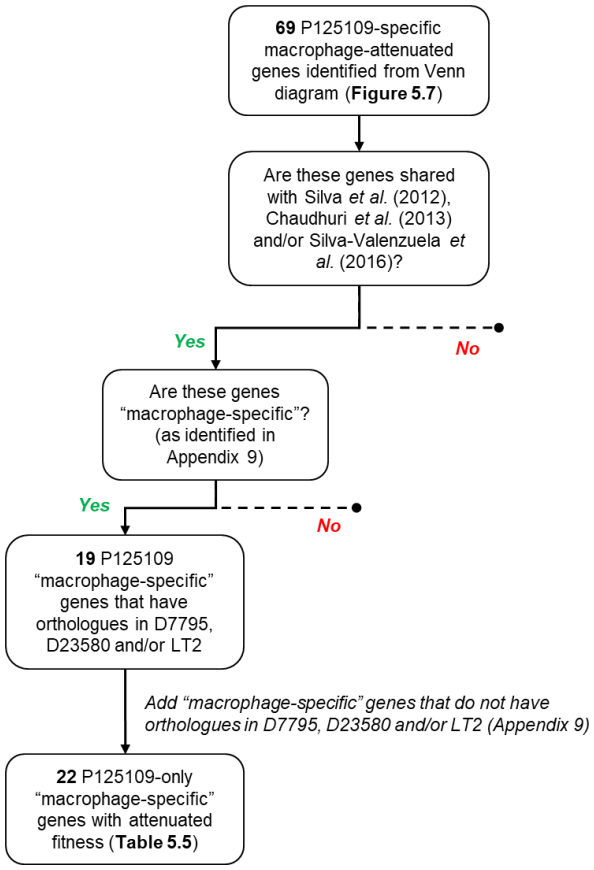

Figure 5.9 Flow chart showing the identification of *S.* Enteritidis P125109-only "macrophage-specific" genes

A similar sequence of comparisons was done with *S.* Enteritidis D7795 to identify D7795-only "macrophage-specific" genes, using **Appendix 11** for information on "macrophage-specific" genes.

Table 5.5 The 22 *S.* Enteritidis P125109-only genes attenuated in macrophage infection

Name	Product	P125109 locus tag	Location	Begin	End	Strand	SEN identifier	D7795 identifier	D23580 identifier	LT2 identifier	P125109 log_2FC	Adj. *P* value
P125109 genes with no orthologues in D7795, D23580 or LT2												
SEN0912	hypothetical protein	P125109_01026	Chr	1014661	1015287	+	SEN0912	NONE	NONE	NONE	−1.41	4.54E−02
tRNA-Ala	tRNA-Ala(ggc)	P125109_02722	Chr	2528346	2528421	−	#N/A	#N/A	#N/A	#N/A	−2.28	8.00E−04
tRNA-Thr	tRNA-Thr(ggt)	P125109_04470	Chr	4232859	4232934	+	#N/A	#N/A	#N/A	#N/A	−3.47	9.82E−03
P125109 genes with orthologues present in D7795, D23580 and/or LT2												
tpke11	NONE	P125109_00013	Chr	13508	13592	+	NONE	D7795_00014	STMMW_ncRNA_1	NONE	−3.32	1.90E−06
yabC	conserved hypothetical protein	P125109_00141	Chr	140329	141270	+	SEN0121	D7795_00143	STMMW_01261	STM0120	−1.23	4.20E−03
yaeD	conserved hypothetical protein	P125109_00284	Chr	293490	294056	+	SEN0256	D7795_00285	STMMW_02532	STM0248	−5.05	3.80E−06
SEN0328	hypothetical protein	P125109_00373	Chr	369042	369530	+	SEN0328	D7795_00374	STMMW_04151	STM0345	−2.14	3.30E−05
SEN0532	hypothetical protein	P125109_00595	Chr	591950	592261	−	SEN0532	D7795_00602	STMMW_06191	STM0551	−1.64	3.80E−03
sucD	succinyl-CoA synthetase alpha chain	P125109_00774	Chr	766695	767564	+	SEN0689	D7795_00799	STMMW_07961	STM0739	−1.35	5.90E−05
SEN0907	putative ion:amino acid symporter	P125109_01022	Chr	1010706	1012142	+	SEN0907	D7795_01047	STMMW_10141	STM1003	−1.01	6.20E−04
yedD	putative lipoprotein	P125109_01175	Chr	1142375	1142788	+	SEN1044	D7795_01257	STMMW_19441	STM1964	−3.78	1.50E−02
cheZ	chemotaxis protein CheZ	P125109_01224	Chr	1183025	1183672	+	SEN1088	D7795_01307	STMMW_18981	STM1915	−1.81	1.80E−03
lrhA	NADH dehydrogenase operon transcriptional regulator	P125109_02623	Chr	2434413	2435351	−	SEN2312	D7795_02672	STMMW_23521	STM2330	−1.57	3.50E−15
iagB	cell invasion protein	P125109_03091	Chr	2906431	2906913	+	SEN2719	D7795_03189	STMMW_28391	STM2877	−2.45	4.20E−03
ygbK	conserved hypothetical protein	P125109_03136	Chr	2944443	2945705	−	SEN2756	D7795_03234	STMMW_28801	STM2917	−1.59	1.90E−02
sORF75	NONE	P125109_03192	Chr	3003598	3003849	+	NONE	D7795_03290	STMMW_29261	NONE	−1.75	2.00E−02

Table 5.5 *(continued)* The 22 *S.* Enteritidis P125109-only genes attenuated in macrophage infection

Name	Product	P125109 locus tag	Location	Begin	End	Strand	SEN identifier	D7795 identifier	D23580 identifier	LT2 identifier	P125109 log_2FC	Adj. *P* value
***(continued)* P125109 genes with orthologues present in D7795, D23580 and/or LT2**												
yggT	putative membrane protein	P125109_03353	Chr	3149863	3150429	+	SEN2944	D7795_03449	STMMW_30621	STM3101	**−1.50**	6.30E−13
glpR	glycerol-3-phosphate regulon repressor	P125109_03805	Chr	3567254	3568012	−	SEN3348	D7795_03904	STMMW_35121	STM3523	**−1.25**	9.50E−12
prlC	oligopeptidase A	P125109_03882	Chr	3645031	3647073	−	SEN3417	D7795_03981	STMMW_35831	STM3594	**−1.28**	3.50E−13
wecG	probable UDP-N-acetyl-D-mannosaminuronic acid transferase	P125109_04235	Chr	4009099	4009839	+	SEN3734	D7795_04333	STMMW_39041	STM3929	**−1.26**	7.20E−08
serB	putative phosphoserine phosphatase	P125109_04914	Chr	4661644	4662612	+	SEN4334	D7795_05011	STMMW_45211	STM4578	**−1.38**	5.80E−06
yjjY	conserved hypothetical protein	P125109_04937	Chr	4684602	4684742	+	SEN4355	D7795_05034	STMMW_45421	STM4599	**−2.03**	3.10E−02

The definition of *S.* Enteritidis P125109-only genes is given in Section 5.2.5.5. Colour scale is the same as shown in **Figure 5.8**.

Table 5.6 The 39 *S.* Enteritidis D7795-only genes attenuated in macrophage infection

Name	Product	D7795 locus tag	Location	Begin	End	Strand	SEN identifier	P125109 identifier	D23580 identifier	LT2 identifier	D7795 log_2FC	Adj. *P* value
D7795 genes with no orthologues in P125109, D23580 or LT2												
group_972	tail fiber assembly protein	D7795_02043	Chr	1895442	1895975	+	NONE	NONE	NONE	NONE	**−1.33**	2.29E−05
tRNA-Arg	tRNA-Arg(cct)	D7795_02738	Chr	2528880	2528954	+	NONE	#N/A	#N/A	#N/A	**−1.64**	4.32E−02
tRNA-Arg	tRNA-Arg(acg)	D7795_03122	Chr	2918004	2918080	−	NONE	#N/A	#N/A	#N/A	**−3.08**	8.28E−03
tRNA-Gly	tRNA-Gly(gcc)	D7795_04774	Chr	4515350	4515425	+	NONE	#N/A	#N/A	#N/A	**−5.59**	3.66E−03
group_1609	hypothetical protein, partial	D7795_05141	pSEN-BT	74086	74565	−	NONE	NONE	NONE	NONE	**−1.28**	4.91E−04
D7795 genes with orthologues present in D7795, D23580 and/or LT2												
yacC	conserved hypothetical protein	D7795_00194	Chr	198136	198522	−	SEN0172	P125109_00193	STMMW_01731	STM0167	**−1.33**	2.30E−02
SEN0286	transposase (fragment)	D7795_00327	Chr	328049	328180	+	SEN0286	P125109_00326	STMMW_03133	NONE	**−1.24**	1.90E−02
queA	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	D7795_00442	Chr	441625	442689	+	SEN0387	P125109_00436	STMMW_04741	STM0404	**−1.26**	1.70E−03
tgt	queuine tRNA-ribosyltransferase%3B tRNA-guanine transglycosylase	D7795_00443	Chr	442886	444013	+	SEN0388	P125109_00437	STMMW_04751	STM0405	**−1.64**	2.10E−13
lipB	lipoate-protein ligase B (lipoate biosynthesis protein B)	D7795_00703	Chr	684667	685308	−	SEN0604	P125109_00677	STMMW_07001	STM0635	**−4.97**	2.10E−05
phoL	PhoH-like ATP-binding protein	D7795_00738	Chr	720603	721649	−	SEN0638	P125109_00713	STMMW_07341	STM0669	**−1.52**	3.10E−06
nagC	N-acetylglucosamine repressor	D7795_00752	Chr	729058	730278	−	SEN0646	P125109_00727	STMMW_07401	STM0682	**−1.02**	3.20E−04
sopD2	putative sopD2 type III secretion system effector protein	D7795_01014	Chr	984727	985686	+	SEN0876	P125109_00989	STMMW_09831	STM0972	**−2.06**	1.60E−02

Table 5.6 *(continued)* The 39 *S.* Enteritidis D7795-only genes attenuated in macrophage infection

Name	Product	D7795 locus tag	Location	Begin	End	Strand	SEN identifier	P125109 identifier	D23580 identifier	LT2 identifier	D7795 log_2FC	Adj. *P* value
***(continued)* D7795 genes with orthologues present in D7795, D23580 and/or LT2**												
pipC	cell invasion protein	D7795_01153	Chr	1109466	1109807	−	SEN0954	P125109_01070	STMMW_11021	STM1090	−1.12	5.50E−03
fliD	flagellar hook associated protein (FliD)	D7795_01261	Chr	1198686	1200092	−	SEN1048	P125109_01179	STMMW_19401	STM1960	−1.27	1.20E−10
fliC	flagellin	D7795_01262	Chr	1200348	1201865	+	SEN1049	P125109_01180	STMMW_19381	STM1959	−1.17	9.70E−17
motB	motility protein B	D7795_01300	Chr	1228685	1229614	+	SEN1081	P125109_01217	STMMW_19051	STM1922	−1.19	5.20E−04
flhA	flagellar biosynthesis protein FlhA	D7795_01310	Chr	1238522	1240600	+	SEN1090	P125109_01227	STMMW_18961	STM1913	−1.14	1.00E−06
rnfE	putative electron transport complex protein rnfE	D7795_01882	Chr	1757120	1757812	+	SEN1593	P125109_01796	STMMW_14571	STM1454	−1.89	7.20E−03
ydiV	conserved hypothetical protein	D7795_01999	Chr	1863606	1864319	−	SEN1700	P125109_01912	STMMW_13511	STM1344	−1.24	4.20E−05
SEN2359	conserved hypothetical protein	D7795_02722	Chr	2510934	2511590	−	SEN2359	P125109_02673	STMMW_23991	STM2377	−1.03	2.20E−02
hscB	Co-chaperone protein hscB	D7795_02906	Chr	2709548	2710063	−	SEN2520	P125109_02858	STMMW_25571	STM2540	−1.98	4.00E−03
pphB	possible serine/threonine protein phosphatase	D7795_03219	Chr	2997269	2997925	+	SEN2745	P125109_03121	STMMW_28701	STM2907	−1.28	2.20E−06
icc	conserved hypothetical protein	D7795_03541	Chr	3299425	3300252	−	SEN3026	P125109_03444	STMMW_31431	STM3183	−1.27	3.00E−10
gatR	galactitol utilization operon repressor	D7795_03624	Chr	3378427	3379200	+	SEN3097	P125109_03525	STMMW_32621	STM3262	−1.16	5.20E−06
SraG	NONE	D7795_03646	Chr	3398395	3398565	−	NONE	P125109_03547	STMMW_ncRNA_221	NONE	−1.57	2.60E−03
greA	transcription elongation factor	D7795_03664	Chr	3414604	3415080	−	SEN3132	P125109_03565	STMMW_32981	STM3299	−1.18	6.50E−03
damX	DamX protein	D7795_03865	Chr	3585221	3586498	−	SEN3311	P125109_03766	STMMW_34751	STM3485	−1.29	4.80E−08

Table 5.6 *(continued)* The 39 *S.* Enteritidis D7795-only genes attenuated in macrophage infection

Name	Product	D7795 locus tag	Location	Begin	End	Strand	SEN identifier	P125109 identifier	D23580 identifier	LT2 identifier	D7795 log_2FC	Adj. *P* value
***(continued)* D7795 genes with orthologues present in D7795, D23580 and/or LT2**												
mtlR	mannitol operon repressor (mannitol repressor protein)	D7795_04081	Chr	3823828	3824418	+	SEN3509	P125109_03982	STMMW_36751	STM3687	−1.18	1.60E−07
asnA	asparagine synthetase A	D7795_04282	Chr	4019283	4020275	+	SEN3691	P125109_04183	STMMW_38611	STM3877	−2.93	1.20E−52
yneB	putative aldolase	D7795_04494	Chr	4219971	4220846	+	SEN3868	P125109_04397	STMMW_40431	STM4078	−1.13	1.50E−18
yjaG	conserved hypothetical protein	D7795_04594	Chr	4321773	4322363	+	SEN3955	P125109_04498	STMMW_41221	STM4169	−1.06	1.00E−03
dgkA	diacylglycerol kinase	D7795_04646	Chr	4379629	4379997	+	SEN4005	P125109_04550	STMMW_41861	STM4236	−1.53	1.00E−04
yjfI	conserved hypothetical protein	D7795_04792	Chr	4533387	4533791	+	SEN4136	P125109_04696	STMMW_43141	STM4370	−1.59	2.90E−02
yjfO	putative exported protein	D7795_04801	Chr	4540618	4540920	−	SEN4145	P125109_04705	STMMW_43231	STM4379	−1.24	3.20E−02
pepA	cytosol aminopeptidase	D7795_04888	Chr	4619190	4620701	−	SEN4230	P125109_04792	STMMW_44231	STM4477	−1.03	2.60E−11
repA2	CDO50996.1	D7795_05036	pSEN-BT	88	1077	+	SEN_p0018	P125109_04939	SLT-BT0121	PSLT023	−1.97	3.00E−04
pefB	CDO50994.1	D7795_05038	pSEN-BT	2673	2975	+	SEN_p0016	P125109_04941	SLT-BT0111	PSLT019	−1.30	1.40E−03
traY	CDO51040.1	D7795_05058	pSEN-BT	20044	20271	+	SEN_p0063	P125109_04972	SLT-BT0801	PSLT076	−1.82	5.70E−03

The definition of *S.* Enteritidis D7795-only genes is given in Section 5.2.5.5. Colour scale is the same as shown in **Figure 5.8**.

5.3 Discussion

African *S.* Enteritidis D7795 is associated with iNTS disease, and displays higher levels of intra-macrophage replication than the Global Epidemic strain P125109 (**Figure 5.2**). To investigate the molecular basis of the observed differences in intra-macrophage replication levels between P125109 and D7795, TIS was used to determine the genetic requirements for macrophage infection. I put the findings into a broader context by doing a direct comparison with our recent publication on the genetic requirements for macrophage infection by African *S.* Typhimurium D23580, and by comparing relevant genes with the results of three other *S.* Enteritidis / *S.* Typhimurium virulence studies involving murine infection. The findings confirmed that many of the *Salmonella* genes required for intra-macrophage replication of *S.* Enteritidis are also important for whole-animal infection, and also highlighted interesting inter-strain differences.

Analysis of transposon mutants associated with the five major SPI regions showed that SPI-2 mutants had a common pattern of attenuation in macrophage infection for *S.* Enteritidis P125109, *S.* Enteritidis D7795 and *S.* Typhimurium D23580 (**Figure 5.8**). The intra-macrophage attenuated mutants corresponded to the SPI-2 region that encodes structural components of the TTSS-2, including SsaV, SseB, SseC and SseD. Following the discovery of SPI-2 (Hensel *et al.*, 1995), this pathogenicity island has been shown to play a key role in *Salmonella* intra-macrophage survival and replication (reviewed in Fàbrega and Vila, 2013 and Petersen and Miller, 2019). Similarly, the SPI-3-encoded MgtC was important for intra-macrophage fitness for P125109, D7795 and D23580; *mgtC* is known to be required for normal *Salmonella* growth in low Mg^{2+} conditions and intra-macrophage survival of *S.* Typhimurium and *S.* Typhi (Alix and Blanc-Potard, 2007; Retamal *et al.*, 2009), and important for *S.* Enteritidis during systemic mice infection (Silva *et al.*, 2012).

SPI-1 plays a crucial role in the invasion of host non-phagocytic epithelial cells by *Salmonella* (Lou *et al.*, 2019), and SPI-1 mediated macrophage entry has also been reported (Drecktrah *et al.*, 2006). Unlike the Drecktrah *et al.* study, which used bacteria grown to late log phase to maximise SPI-1 expression, the *S.* Enteritidis transposon library cultures were grown overnight to stationary phase where SPI-1 expression is minimal. Additionally, the bacterial cultures used for infection were opsonised with normal mouse serum (Section 2.5.2). Macrophage entry by the *S.* Enteritidis transposon mutants is therefore expected to be mainly via phagocytosis, and the findings reflect that there was minimal contribution of the SPI-1 genes in macrophage infection. However, SPI-1-located *iagB* (encoding a cell invasion protein) and *hilC* (encoding a transcriptional regulator of SPI-1 expression) genes were identified as required for intra-macrophage fitness in P125109 but not D7795 and D23580 (**Figure 5.8**). I note that a requirement of *iagB* was also reported in *S.* Typhimurium SL1344 but not *S.* Typhi for infection of THP-1 macrophages (Langridge, 2010). Overall, I conclude that the lack of attenuation associated with the SPI-1 genes suggests that the TTSS-1 system is not required for the intra-macrophage fitness of both *S.* Enteritidis strains.

Of the 38 genes encoding known and candidate *Salmonella* effector proteins analysed in this study, the SPI-2 TTSS effectors SseF and SifA showed a consistent phenotype of being required for fitness of P125109, D7795 and D23580 in macrophage infection (**Table 5.2**). SseF and SifA belong to a core set of effector proteins present in different *Salmonella* serovars, and contribute to the maintenance of the SCV (Jennings *et al.*, 2017), the crucial intracellular environment where the bacteria replicates.

Mutants in the LPS biosynthesis genes (*rfaHIJKL*, *rfbCDKMNP* and *galE*) had increased intra-macrophage fitness in both *S.* Enteritidis strains (**Appendix 13L**), in

agreement with observations from Canals *et al*. (2019a) and Venturini *et al.* (2020), but contrary to observations from animal infection studies (e.g. Silva *et al.*, 2012; Chaudhuri *et al.* 2013; Silva-Valenzuela *et al.*, 2016). The *rfb* genes and *galE* are involved in O-antigen synthesis while the *rfa* genes mediate LPS core synthesis (Schnaitman and Klena, 1993). As Canals *et al.* (2019a) noted, *Salmonella* mutants lacking the LPS O-antigen have been shown to be phagocytosed at higher levels than wild-type strains by murine macrophages (Hölzer *et al.*, 2009). This likely accounts for the increased number of *S.* Enteritidis LPS mutants recovered in the macrophage output, and also highlights the importance of interpreting the results of a mutant screen in the context of the experimental model used.

Some genes with orthologues present in all three *Salmonella* strains displayed a strain-specific requirement for fitness in macrophage infection. For example, transposon insertions in the genes encoding SPI-2 effectors *spvD* and *sopD2*, and the SPI-1 effector *sopF* resulted in significant levels of attenuation in D7795 but not in P125109 and *S.* Typhimurium D23580 (**Table 5.2**).

The importance of SpvD for *S.* Typhimurium pathogenesis has been reported previously: SpvD, a cysteine hydrolase, is an inhibitor of the NF-κB proinflammatory pathway (Grabe *et al.*, 2016; Rolhion *et al.*, 2016). Amino acid polymorphisms exist between the SpvD proteins of *S.* Enteritidis and *S.* Typhimurium, with the D7795 variant having the highest enzymatic activity, largest anti-inflammatory effect and greater virulence *in vivo* (Grabe *et al.*, 2016). SopD2 interferes with host endocytic trafficking to lysosomes (D'Costa *et al.*, 2015), and SopF promotes stability of nascent SCVs and also inhibit bacterial autophagy by the host cell (Lau *et al.*, 2019; Xu *et al.*, 2019). These reports suggest that the ability to evade host defence systems could be

a key virulence feature of D7795, as it is for both *S.* Typhi and *S.* Typhimurium (Bernal-Bayard and Ramos-Morales, 2018).

Conversely, the SPI-5 encoded SPI-2 effector PipB was shown to be required by P125109 only but not D7795 and D23580 for intra-macrophage fitness (**Table 5.2**). PipB has previously been shown to be associated with SCVs, but does not contribute to SCV formation and maintenance, nor affect intracellular replication of *S.* Typhimurium in epithelial and macrophage cells (Knodler *et al.*, 2002). Interestingly, both *sopD2* and *pipB* are highly expressed during macrophage infection of D23580 (Canals *et al.*, 2019b), but neither protein is required for the intra-macrophage fitness of D23580.

My findings that certain effector proteins are required for intra-macrophage fitness while others are dispensable, leads me to speculate some strain-specific functions or interactions are performed by the effectors resulting in different virulence phenotypes. However, to develop this idea further, experimental validation will be needed to ascertain that these specific genes are genuinely required for intra-macrophage fitness of the bacterial strain in question.

The key discovery reported in this chapter is the list of candidate *S.* Enteritidis virulence genes highlighted in **Table 5.5** and **Table 5.6**. Majority of the genes have orthologues present in other *Salmonella* strains or serovars that are not required for intra-macrophage fitness, reinforcing the earlier speculation that the proteins are performing strain-specific functions. Intriguingly, many of the P125109-only and D7795-only “macrophage-specific” genes are tRNA genes. tRNA genes are known to be hotspots for the integration of mobile genetic elements such as SPIs (Hensel, 2009), but a role in regulating *Salmonella* virulence has also been reported e.g. the control of the *mgtCBR* operon by $tRNA^{Pro}$ (Lee *et al.*, 2014). Constructing individual

gene deletions and using these mutants for detailed phenotypic and virulence experiments should unveil some hitherto unknown *Salmonella* virulence factors.

Overall, the TIS technique represents an improvement to previous *S.* Enteritidis mutagenesis screens. The Silva *et al.* (2012) study assayed a total of 54,000 mutants in P125109, while the Shah *et al.* (2012) study assayed 4,992 mutants. Because the TIS experiments described here involved between 140,000 and 190,000 individual transposon mutants, it was possible to sample regions that were missed in previous screens e.g. Silva *et al.* (2012) noted that their observations did not identify *yfgL* (Fardini *et al.*, 2007) and *yhbC* (Chang *et al.*, 2008), both of which have been shown to play a role in chick virulence and were also found to be important for intra-macrophage fitness here (**Appendix 9** and **Appendix 11**). However, my findings were not completely comprehensive e.g. genes from the ROD9 region (SPI-19) were not consistently identified, even though a virulence role of several ROD9 mutants (e.g. *SEN1001*, *SEN1005* and *SEN1008*) in cell culture and animal infection models have been established (Das *et al.*, 2018; Das *et al.*, 2020; Silva *et al.*, 2012). Nonetheless, I have identified a role in intra-macrophage fitness for many genes known to be fundamental to *Salmonella* virulence in both *S.* Enteritidis P125109 and D7795.

Notably, the results reported here document the first large-sale mutagenesis-based analysis of African *S.* Enteritidis. The constructed transposon libraries in African *S.* Enteritidis strain D7795 can now be used for more comparative analyses with Global Epidemic clade *S*. Enteritidis as well as other *Salmonella* serovars, and enhance our understanding of African *Salmonella* biology.

Chapter 6 :

General Discussion

6.1 Importance of this study

6.1.1 Context of the study

S. enterica subsp. *enterica* serovars are important pathogens responsible for three distinct disease syndromes: enteric fever, gastroenteritis, and bloodstream infection. *S.* Typhimurium and *S.* Enteritidis are the two most common NTS serovars responsible for the majority of salmonellosis infections in humans worldwide (Section 1.1). In recent years, genetic variants of *S.* Typhimurium and *S.* Enteritidis that are highly associated with bloodstream infections have been identified in sub-Saharan Africa (Feasey *et al.*, 2016; Kingsley *et al.*, 2009; Okoro *et al.*, 2012). Characterisation of these iNTS-infection associated strains revealed that sub-Saharan African isolates had specific genomic signatures that included multi-drug resistance determinants, distinct prophage repertoires and distinct patterns of genome degradation (Feasey *et al.*, 2016; Kingsley *et al.*, 2009; Pulford *et al.*, 2020). In contrast to African *S.* Typhimurium, which has been an active focus of research in the past decade, research involving African *S.* Enteritidis has lagged behind.

Overall, the genetic determinants mediating the enhanced propensity of African *Salmonella* to cause systemic infection compared to gastroenteritis-associated *Salmonella* remain unclear. This study set out to investigate the genetic requirements of gastroenteritis-associated and iNTS infection-associated African clades of *S.* Enteritidis, in terms of *in vitro* growth and macrophage infection, using the reference strains P125109 (Global Epidemic clade) and D7795 (Central/Eastern African clade). Two different functional genomic approaches were considered during this project (RNA-seq and TIS); TIS was chosen to address the research question as it proved to be the most tractable approach.

6.1.2 Key findings and their significance

RNA-seq-based transcriptomics has proved to be a popular and useful approach for defining host-pathogen interactions of *Salmonella* (reviewed in Colgan *et al.*, 2017; Westermann and Vogel, 2021), and for understanding *Salmonella* gene function (Perez-Sepulveda and Hinton, 2019). However, the use of RNA-seq to determine the gene expression of *Salmonella* during infection has been hampered by the challenge of the isolation of bacterial RNA from infected host cells (Hinton *et al.*, 2004; La *et al.*, 2008; Skvortsov and Azhikina, 2010). Although new developments in RNA-seq technology now enable simultaneous transcriptomic analysis of host and pathogen, the actual cost of the depth of sequencing required to report an accurate bacterial transcriptome has proved to be prohibitive for many research laboratories. This dual-RNA-seq approach has been reviewed by Westermann and Vogel (2021).

In Chapter 3, I described a set of experiments to optimise an intracellular *Salmonella* RNA extraction protocol developed by the Hinton Laboratory and which has been used successfully to study the *S.* Typhimurium transcriptome during infection of epithelial cells (Hautefort *et al.*, 2008) and macrophages (Canals *et al.*, 2019b; Eriksson *et al.*, 2003; Srikumar *et al.*, 2015), which focused on improving the separation of *Salmonella* cells from lysed macrophages. Of the modifications tested, lysate homogenisation using needle and syringe proved to be the most promising for improving the recovery of *Salmonella* cells from macrophage lysates. However, a concomitant increase in the yield of intra-macrophage *Salmonella* RNA yield proved to be unreproducible. Despite the inconclusive findings from the optimisation experiments, my observations will inform future users of the intracellular *Salmonella* RNA extraction protocol of potential pitfalls to be avoided.

In light of the technical difficulties associated with intra-macrophage *Salmonella* RNA extraction, Chapters 4 and 5 report findings from a second functional genomics

approach, TIS. I used TIS to investigate the genetic requirements for *S.* Enteritidis P125109 and D7795 for both *in vitro* growth and macrophage infection. TIS is a high-throughput genome-wide screening approach that has been successfully used to identify genes of various bacteria species that play functional roles in different environmental conditions. To our knowledge, this is the first report of the TIS-based identification of genetic requirements of *S.* Enteritidis strains P125109 and D7795 for *in vitro* growth and macrophage infection.

In Chapter 4, the *S.* Enteritidis transposon libraries were analysed prior to experimental selection and following selection in LB, NonSPI2 and InSPI2 media. I determined that 497 P125109 genes and 467 D7795 genes were required for growth under laboratory conditions (Section 4.2.1). Comparing the overlap between required genes in the respective *S.* Enteritidis strains with gene sets derived from *S.* Typhimurium, *S.* Typhi, and *E. coli* identified 207 genes in common (Section 4.2.1). The involvement of the 207 genes in vital cellular processes such as DNA replication, transcription and translation effectively validated the TIS screening approach for identifying required genes of *S*. Enteritidis.

To overcome the challenges of analysing transposon libraries that had very different densities, a custom pipeline was used to compare the *in vitro* required gene sets between P125109, D7795 and the African *S.* Typhimurium strain D23580, which is the representative *S.* Typhimurium strain associated with iNTS infection. The resulting 63 genes that were differentially required between the three strains, show a clear distinction between the serovar-specific genes and African *Salmonella*-specific genes required for *in vitro* growth (**Figure 4.7**). Distinct genetic requirements for optimal growth in three different growth media (LB, NonSPI2 and InSPI2) were also determined for P125109 and D7795. In future, a thorough exploration of these genetic

differences is likely to reveal more about the functions that underpin the lifestyles of the two bacterial strains.

In Chapter 5, experiments comparing the intra-macrophage replication between Global Epidemic clade *S.* Enteritidis and Central/Eastern African clade *S.* Enteritidis confirmed that Central/Eastern African *S.* Enteritidis are more virulent than Global Epidemic clade during infection of mammalian cells *in vitro* (**Figure 5.2**). I then investigated the genes that contribute to fitness during macrophage infection in P125109 and D7795, and showed that many well-characterised *Salmonella* virulence genes are important for macrophage infection in both *S.* Enteritidis strains. A key discovery is the list of 22 candidate P1251095-specific and 39 D7795-specific virulence genes that were required for optimal macrophage infection (**Table 5.5** and **Table 5.6**). Three P125109 genes and five D7795 genes had no orthologues in the other *Salmonella* strains referenced in this study, and have the potential to encode novel virulence factors. In future, experimental validation by knockout deletion is needed to determine whether these genes do impact upon intra-macrophage fitness.

The candidate D7795-specific virulence genes include several effector proteins that have immunomodulatory properties on macrophage functions (SpvD, SopF, SopD2), raising the possibility that escaping the macrophage innate immune response accounts for the observed higher levels of intra-macrophage replication in D7795 (**Figure 5.2**). Indeed, immune evasion is a common theme in *Salmonella* pathogenesis (reviewed by Behnsen *et al.*, 2015; Bernal-Bayard and Ramos-Morales, 2018). As discussed in Chapter 1, examples of immune evasion strategies include the loss or reduced expression of flagellin, which is recognised by host TLR5 and NLRC4 receptors to trigger an inflammatory response, and cause reduced cytotoxicity in infected host cells. The ability to evade the mammalian immune system has been reported in host-restricted serovars such as *S.* Typhi, *S.* Paratyphi and

S. Gallinarum (Atif *et al.*, 2014; de Freitas Neto *et al.*, 2013; Winter *et al.*, 2010b; Winter *et al.*, 2015), as well as African *S.* Typhimurium ST313 strains (Ramachandran *et al.*, 2015), and *S.* Enteritidis (Kilroy *et al.*, 2017). Immune evasion facilitates disseminated infection and host colonisation by this diverse range of *Salmonella* serovars (Kurtz *et al.*, 2017).

Another example of immune evasion is mediated by the over-expression of the PgtE protease in African *S.* Typhimurium ST313, which confers resistance to complement-mediated killing in human serum (Hammarlöf *et al.*, 2018). It is hoped that the search for bespoke immune evasion strategies of African *S.* Enteritidis D7795 will reveal the underlying molecular basis for the enhanced intra-macrophage replication phenotype that I observed (Section 5.2.1).

6.2 Future prospects

The establishment of TIS for the investigation of gene function in African *S.* Enteritidis will facilitate several lines of research. Besides the Central/Eastern African clade, there is also the Western African clade of *S.* Enteritidis which is less well-characterised than Central/Eastern African clade *S.* Enteritidis. Both Poulsen *et al.* (2019) and Rousset *et al.* (2021) have demonstrated the value of including multiple strains and/or growth conditions for the investigation of genetic requirements. This thesis only examined three infection-relevant *in vitro* conditions and intra-macrophage replication in murine macrophages. If further resources could be invested, it would be of great interest and relevance to explore the genetic requirements of *S*. Enteritidis from both African clades in additional environmental conditions.

A major, but inevitable, limitation of this study was the lack of experimental validation, due to the restrictions imposed on research activities by the ongoing COVID-19 pandemic. To make concrete deductions, genes identified in a TIS screen need to be

validated by knockout deletions. Given that a TIS screen can potentially yield hundreds of gene hits, deciding which gene(s) to target for follow-up validation can be challenging. The integration of TIS findings with transcriptomic data can help inform this decision, by ensuring that the genes targeted are biologically relevant. For example, Canals *et al.* (2019a) showed that genes disrupted by transposon insertion that resulted in significant attenuation of macrophage infection were also highly upregulated during macrophage infection (Canals *et al.*, 2019b). Transcriptomic analyses of P125109 and D7795 under infection-relevant conditions are already under way and will serve as important complementary datasets to the TIS data generated in this study.

Ideally, the study of host-pathogen interactions should be multi-faceted. While TIS offers information about genes required for the experimental condition investigated, it does not unambiguously confirm the involvement of the gene (Langridge, 2010). The integration of data from various "-omics" experiments is needed to provide a fuller picture of the molecular interactions at play. For example, a recent study by Venturini *et al.* (2020), combined the use of dual RNA-seq (transcriptional evidence), Ribo-seq (translational evidence) and Grad-seq (protein evidence) with TraDIS (genetic evidence) to investigate the contribution of small proteins in *S.* Typhimurium virulence, and uncovered a new role for the small protein MgrB.

6.3 Concluding remarks

Overall, this study has demonstrated that gastroenteritis-associated *S.* Enteritidis P125109 and bloodstream infection-associated African *S.* Enteritidis D7795 share many similarities in genetic requirements for *in vitro* growth and macrophage infection, that have also been identified in other *Salmonella* serovars. Both *S.* Enteritidis strains encode candidate virulence genes that were important for fitness during macrophage infection. However, D7795 did not share novel virulence factors

with African *S.* Typhimurium D23580 that were absent from gastroenteritis-associated *Salmonella*.

It is hoped that the publication of the TIS datasets generated in this study will serve as a useful community resource for researchers to formulate functional hypotheses and investigate genes of interest. Given the recent advances in various "-omics" technologies and increasing availability of global datasets, I anticipate that the analysis of my TIS-based fitness data with other datasets yet to be generated will yield new insights into the molecular differences responsible for the different pathogenic lifestyles of gastroenteritis-associated *S.* Enteritidis and bloodstream infection-associated African *S.* Enteritidis.

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